Plant morphogenesis and genetic transformation of horticultural brassicas

Thesis

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PLANT MORPHOGENESIS AND GENETIC TRANSFORMATION OF HORTICULTURAL BRASSICAS

By

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Abstract

This study set out to elucidate the genetic control of *in vitro* shoot morphogenesis and the production of transgenic plants in horticultural brassicas. Desirable characteristics associated with successful transformation were identified. These include susceptibility to *A. tumefaciens,* high shoot regeneration potential and the production of multiple shoots in association with a distinct swelling or callus phase. Diallel analysis showed that both high and multiple shoot regeneration are heritable traits, with additive gene effects accounting for 71 % and 77 % of the genetic variation respectively. High susceptibility to *A. tumefaciens* was shown to have a positive effect on transformation efficiency. A highly significant putative QTL associated with susceptibility to *A. tumefaciens* was located on linkage group O9 of *B. oleracea*, and substitution lines confirmed the presence of this QTL. Additive gene effects also accounted for 79 % of the genetic control of susceptibility to *A. tumefaciens*. The ability to increase both *in vitro* shoot regeneration potential and susceptibility to *A. tumefaciens* in subsequent generations, through conventional breeding methods, was successfully demonstrated. Tissue culture blackening was shown to be a critical factor limiting the production of transgenic shoots.

A *Brassica oleracea* genotype, DH 1012, was identified that produced transgenic shoots at a rate higher than anything previously reported, for the transformation of cotyledonary petioles using *A. tumefaciens*. The speed and efficiency of regeneration enabled the isolation of transgenic shoots just 4 weeks after inoculation with *A. tumefaciens*. The efficacy of DH 1012 as a potential model genotype for transformation is demonstrated. A transformation rate of up to 31 % combined with the availability of an associated genetic linkage map will make this genotype very desirable as a potential research and development tool for studying gene function.

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Chapter 1 General Introduction

1.1 Plant genetic transformation

Conventional breeding programmes, for the improvement of crops such as brassicas, often involve the crossing of two parents followed by several generations of backcrossing to recover the desired cultivar characteristics. With the development of plant genetic transformation the potential to introduce specific genes of interest directly into a desired cultivar, without the introduction of many undesired traits, became possible. The ability to by-pass sexual incompatibility also enabled plant breeders to widen the gene pool available to them, thus allowing the introduction of genes from unrelated plant species, microorganisms and animals.

Plant genetic transformation has been developed for a large number of plant species, using a wide range of techniques. Despite the great advances in procedures, the efficiency and success of plant transformation remains highly species specific, with protocols often being tailor-made for a particular species or even genotype. This thesis reports on research carried out to investigate the genetic basis of transformation in horticultural brassicas, in order better to understand what governs variation in transformation efficiency. An improved understanding of the causes of variation has led to the identification of genotypes amenable to plant genetic transformation.

1.2 The *Brassica* genus

The genus *Brassica* belongs to the family Cruciferae and includes a group of six inter-related species of worldwide economic importance. *Brassica rapa* (genome AA, 2n = 20) is used both as a vegetable crop (turnip and Chinese cabbage) and as an

oleiferous crop (turnip rape). *B. nigra* (BB, 2n = 16) is grown as a condiment (black mustard) and *B. oleracea* (CC, 2n = 18) contains numerous vegetable crops with a wide range of different morphologies such as cabbage, cauliflower, kale, broccoli and Brussels sprouts. The ancestral hybrids *B. juncea* (AABB, 2n = 36, brown mustard) and *B. napus* (AACC, 2n = 38, oilseed rape) are important oilseed crops and *B. carinata* (BBCC, 2n = 34) is grown in Ethiopia as both a vegetable and oil crop (Ethiopian/Abyssinian mustard). U (1935) established the evolutionary origin of the three amphidiploid species (*B. juncea, B. napus* and *B. carinata*) as a result of cytogenetic analysis of interspecific hybrids between the three diploid species (*B. rapa, B. oleracea* and *B. nigra*). This relationship is expressed diagrammatically in Figure 1.

The genetically diverse array of vegetable and oilseed crops, outlined above, has been the result of years of hybridization between and within the *Brassica* species, accompanied by intense selection for different morphologies. The application of conventional breeding has led to the development of many superior cultivars within this genus. With increasing knowledge of the function of genes, and the development of techniques for plant transformation, the potential for further improvement of these species is considerable.

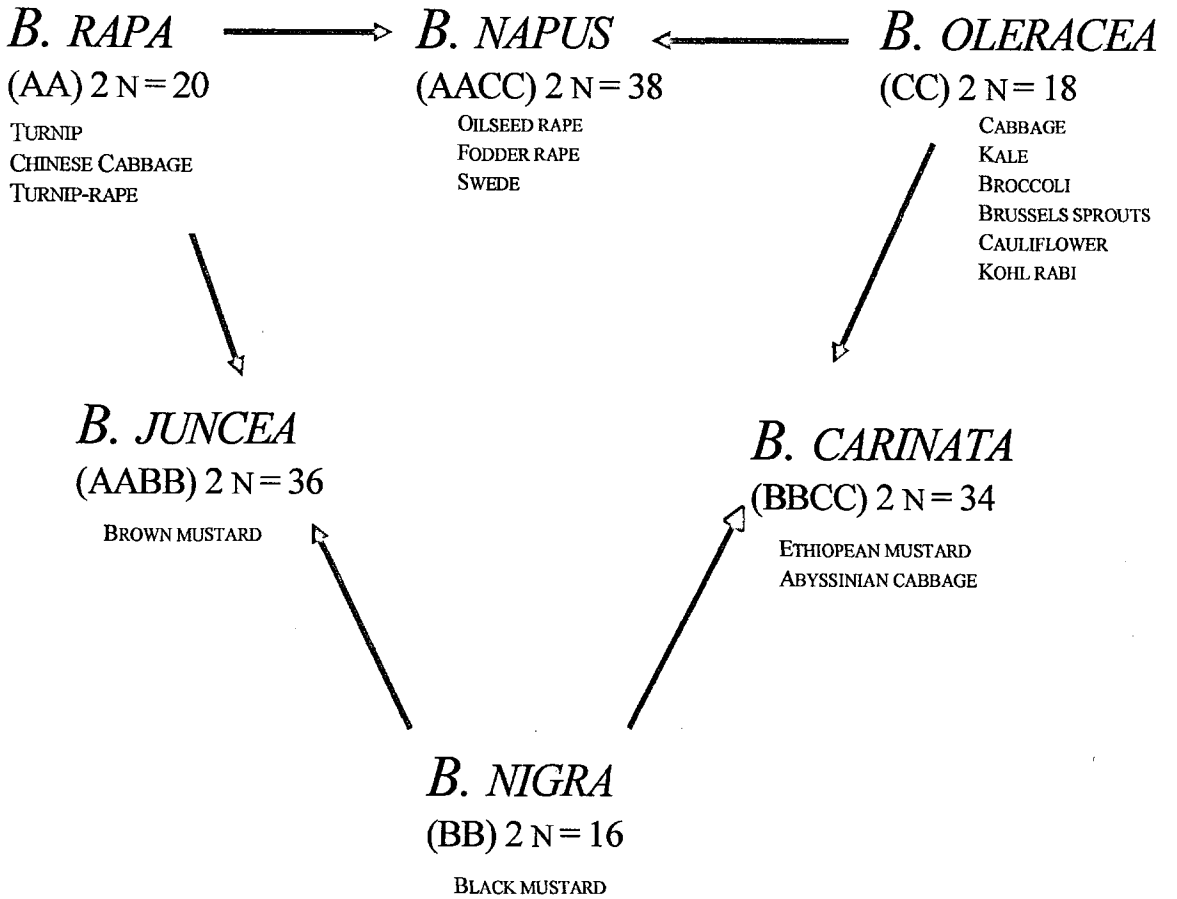


Figure 1 **U's Triangle: The genetic relationship of the cultivated *Brassica* species. Redrawn from U (1935).**

1.3 Transformation of *Brassica*

In order to overcome the limitations of conventional plant breeding, biologists have experimented with various ways of introducing genes through non-sexual methods. Recombinant DNA technology and improved molecular biology techniques have enabled scientists successfully to introduce single genes from related and non-related species into plants. This has led to the production of crops with improved yields, more nutritional and healthier plants, increased plant resistance to pests, diseases, herbicides and environmental stresses, but also the introduction of novel crops capable of producing antibodies or chemicals for use by non-food industries, reviewed by Poulsen (1996) and Puddephat *et al.*, (1996).

Specific gene transfer or genetic 'transformation' (as it will be referred to in this thesis) has been developed for many plant species. It is clear from the range of published protocols that no single universal system works for all species. Transformation protocols and their efficiencies are strongly influenced by plant genotype, with protocols often being tailor-made for particular species or even particular genotypes within a species. For the transformation of *Brassica,* a number of approaches have been developed for the introduction of foreign genes (Table 1).

Table 1 Methods employed for *Brassica* transformation

Brassica Species	Transformation method	Key Reference
B. rapa *B. nigra* *B. oleracea* *B. napus* *B. juncea* *B. carinata*	*Agrobacterium tumefaciens*	Radke *et al.*, 1992 Gupta *et al.*, 1993 De Block *et al.*, 1989 Moloney *et al.*, 1989 Barfield and Pua, 1991 Babic *et al.*, 1998
B. oleracea *B. napus*	*Agrobacterium rhizogenes*	David and Tempe, 1988 Guerche *et al.*, 1987a
B. oleracea *B. nigra* *B. napus*	Direct gene transfer	Mukhopadhyay *et al.*, 1991 Gupta *et al.*, 1993 Guerche *et al.*, 1987 b
B. napus *B. oleracea*	Particle bombardment	Chen and Beversdorf, 1994 Phuddephat *et al.*, 1999

For the *Brassica* genus, *Agrobacterium*-mediated methods of transformation are the most commonly used approach.

1.3.1 *Agrobacterium*-mediated transformation

Agrobacteria are gram-negative rod shaped bacteria present in the soil and rhizosphere. As a general rule, wild-type strains of *Agrobacteria* will infect wounded dicotyledonous species, but monocotyledonous plants, particularly the cereals, have

been considered outside the host range for *Agrobacterium*. There has, however, been renewed interest in using *A. tumefaciens* as a vector to transform economically important grasses and other monocotyledons (Smith and Hood 1995), and significant progress is being made (Nadolska-Orczyk *et al.*, 2000, review). There are two species of *Agrobacterium* that are commonly used for transforming plants, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. Both species cause infection by the transfer of a defined piece of DNA, the transferred DNA or T-DNA from the tumour inducing (Ti) or root inducing (Ri) plasmid of *A. tumefaciens* or *A. rhizogenes* respectively.

1.3.1.1 *Agrobacterium tumefaciens*

In its wild-type form *A. tumefaciens* can enter damaged dicotyledonous plants and cause cell proliferation at the wound site, resulting in the formation of crown galls or tumours. A wide variety of transformation vectors based on the Ti plasmid have been developed. These do not contain the tumour inducing (oncogenic) sequences responsible for crown gall formation, and hence plant regeneration and normal plant growth can be obtained following the transfer of T-DNA into the nucleus of the recipient plant cell. In the engineered form, tumour-inducing genes are replaced with genes of interest and a selectable marker gene. The *A. tumefaciens* then acts as a vector to transfer these genes into the host plant.

1.3.1.2 *Agrobacterium rhizogenes*

In its wild-type form *A. rhizogenes* gives rise to 'hairy root' symptoms rather than crown galls. In the engineered *A. rhizogenes* a separate (introduced) plasmid contains the genes of interest. The resulting transgenic plants contain both the genes of interest

and also the *rol* (root inducing loci) genes of the Ri plasmid. The presence of *rol* genes results in the production of plants with a characteristic altered phenotype (the Ri phenotype); wrinkled leaves, reduced fertility, shortened internodes and reduced apical dominance (Guerche *et al.,* 1987a). The Ri phenotype gives an indication of transformation success without the need for a selectable marker (e.g. antibiotic resistance genes) and the unwanted Ri phenotype can then be segregated away in subsequent sexual generations. Transgenic plants that do not contain antibiotic resistance genes are much more acceptable commercially. However, sometimes both the transgene and the *rol* genes have insertion sites that are so closely linked on the same chromosome that they cannot easily be separated by genetic recombination. The difficulty in some instances, of removing the Ri phenotype (along with potential problems of reduced fertility) may reduce the efficiency of this system.

For the purpose of this thesis the term 'transformation' will refer to *A. tumefaciens* transformation. *A. tumefaciens* has been used to transform all the main *Brassica* species (Table 1) however, the methods vary considerably in transformation efficiency both between and within *Brassica* species.

1.4 Transformation variables

The primary focus of this research was to gain a better understanding of the genetic basis of transformation in the horticultural brassicas, *i.e.* to understand why some genotypes can be transformed while others remain recalcitrant. Within a transformation system there are three stages that are considered to be the main areas responsible for variation in transformation efficiencies. These are:

1. **Shoot morphogenesis:** Regeneration of whole plants from transformed cells.

2. **Susceptibility to *Agrobacterium***: Transfer of T-DNA and expression by the plant host.
3. **Selection methods**: Means of selecting transformed cells.

1.4.1 Shoot morphogenesis (or shoot regeneration)

The ability to transform a plant cell is of little use if whole plant regeneration from this cell is not possible. It is important therefore that every transformation programme should include development of an efficient and reliable plant regeneration system. Many plant cells are believed to contain the complete genetic information needed to enable them to develop into a whole plant. Plant cells are able to differentiate into different cell, tissue and organ types by regulating gene expression. It is possible therefore, for some differentiated cells to become undifferentiated cell types. Cells of dicotyledonous plants, induced by simple wounding, appear to dedifferentiate easily and regain mitotic activity. Given appropriate environmental conditions e.g. hormones and nutrients, these cells can even regenerate whole plants (an ability known as totipotency). In contrast, differentiation of meristematic tissues of monocotyledonous species leads to a rapid decline of mitotic activity and the process quickly becomes difficult, or impossible to reverse.

Plant tissue culturists have exploited the totipotency of plant cells for a range of breeding purposes. These include:

- The maintenance of elite breeding lines in clonal germplasm banks.
- Micropropagation for continuous *in vitro* production of plants without seasonal variation. This can also be used to create virus-free plants, and can be used to propagate plants *in vitro* that are hard or impossible to propagate *in vivo*.

- Microspore or anther culture for example, the production of homozygous doubled haploid lines.
- Transformation.

In vitro methods have contributed to progress in plant breeding, and many of these methods (with the exception of transformation) are currently perceived as part of conventional breeding practices.

The general conditions for shoot regeneration are similar for many species. Seedlings remain the favoured source of tissue for *in vitro* morphogenesis as seeds can be surface sterilised, to prevent contaminants (microorganisms) growing on the culture medium, and thus seedlings can be germinated under sterile conditions. It is thought that embryonic / young tissue is more amenable for dedifferentiation. Older tissue is generally found to be more tightly genetically programmed and can also be difficult to surface sterilise. The research presented in this thesis has concentrated on shoot regeneration from cotyledonary petioles and hypocotyl explants.

The basal culture medium employed for shoot morphogenesis is similar for most *in vitro* protocols. It generally contains a mixture of macro- and micro-nutrients, a carbon source and vitamins, with a pH of between 5 to 6. The main variables among protocols are the type and levels of hormones added. Generally cytokinins favour shoot regeneration whilst auxins favour root formation, though frequently a combination of cytokinin and auxin is required for shoot regeneration. Regeneration can either be direct, or indirect via an initial callus phase. Indirect shoot regeneration may require the use of different media for callus initiation preceding shoot regeneration.

Regeneration among the *Brassica* species has been achieved by a number of approaches. In *B. oleracea* alone, a number of different explant types have been utilised:

1. Leaf disc (Lazzeri and Dunwell, 1986)
2. Protoplasts (Jourdan and Earle, 1989; Hansen and Earle, 1994)
3. Peduncles (Christey and Earle, 1991)
4. Seedling tissues –
    - cotyledons (Moloney, 1989)
    - hypocotyls (Lazzeri and Dunwell, 1986; David and Tempe, 1988)
    - roots (Lazzeri and Dunwell, 1984)

Many of the protocols listed will have been tailored to suit a particular genotype of interest. To date the general approach for improving regeneration has been to (1) evaluate a number of combinations of culture conditions to find one that suits a particular genotype or (2) to develop a system which favours regeneration from the majority of genotypes e.g. Christey and Earle (1991). Both approaches have shown regeneration to be highly genotype dependent (with shoot regeneration efficiencies ranging from 0 to 100 %) and despite the totipotency theory, some lines have remained recalcitrant to *in vitro* tissue culture techniques.

Although extensive screening of culture conditions has improved *Brassica* regeneration, the effect of genotype still overrides most efforts to improve protocols for regeneration. By understanding the genetics behind regeneration it may be possible to select for particular genes or introduce genes for regeneration ability into agronomically elite lines. This approach requires knowledge of the genetics underlying regenerability, including the number of genes involved and gene interactions. Knowledge of simple inheritance patterns of regenerability would facilitate the identification of molecular

markers for shoot regeneration and thus make transfer and selection for the trait, in breeding programmes, easier and faster (Hansen *et al.,* 1999).

1.4.1.1 Genotype dependence of shoot regeneration in *Brassica*

To better understand the genotype dependence of shoot regeneration, a number of research groups have examined variation in regeneration response both between and within the *Brassica* species. Murata and Orton (1987) studied shoot regeneration in 7 *Brassica* species and concluded that genes responsible for enhanced regeneration may be localised on the C genome of *B. oleracea*. They observed that *B. napus* (AACC) had a higher regeneration response than *B. rapa* (AA) thereby concluding that genes from the C genome may be influencing its greater regeneration response. Another study looked at regeneration in the three diploid *Brassica* species and their synthetic amphidiploid hybrids, and in this case the regeneration response of the parents was known. Using this approach Narasimhulu *et al.,* (1988a,b), concluded that the A genome was the most recalcitrant genome for regeneration. Both synthetic *B. napus* (AACC) and *B. juncea* (AABB) gave much lower regeneration responses than the best parental response of *B. oleracea* (CC) and *B. nigra* (BB) respectively, suggesting an inhibitory effect of the A genome. They found no significant difference between the B and C genomes in terms of regeneration potential. These studies suggest that shoot regeneration is heritable.

In summary, the ability to regenerate shoots and the frequency of shoot production is genotype dependent, with variations observed both within and between species. *B. rapa* appears to be the most recalcitrant species (or contains genes that inhibit or do not favour shoot regeneration). Crossing a genome with low capacity for shoot regeneration with a genome with a high capacity produces a hybrid with a regeneration

frequency higher than that of the lower regenerating parental genome, this provides further evidence that shoot regeneration is a heritable trait.

1.4.1.2 The genetic analysis of shoot regeneration

A number of approaches have been employed to identify the genetic control of shoot regeneration. These include:

1. The use of diallel hybridization programmes; where a set of n genotypes are self pollinated and intercrossed in all combinations including reciprocals to produce n^2 families, to study inheritance patterns.
2. Segregating (mapping) populations, e.g. F_1, F_2 and BC populations are useful for identifying inheritance patterns and may identify the number of genes involved (depending on the number of genes and size of the segregating population). Mapping populations that are associated with a genetic map can facilitate the identification of quantitative trait loci (QTL) associated with the genetic variation under investigation.
3. Substitution lines (in their simplest form) involve the creation of two genotypes that are identical apart from a defined region on a particular chromosome. Any genetic differences in phenotype between these two genotypes must be due to genes in this defined region. The smaller the region, the more precisely the position of these genes will be known.

A summary of findings to date is given in Table 2 (page 20). It is apparent that regeneration from different tissue types may be controlled by different genes / loci. Differentiated cells are generally believed to contain all the genetic information to regenerate whole plants, so something happens during differentiation that affects the

capacity of different tissue types to dedifferentiate and subsequently regenerate into a new plant.

1.4.1.3 The genetic analysis of shoot regeneration in *Brassica*

The genetic analysis of shoot regeneration from cotyledonary explants has been studied in *Brassica napus*. Ono and Takahata (2000) carried out a 7 x 7 diallel cross using cultivars showing different abilities for shoot regeneration. The diallel comprised three high and three low regenerating lines and one non-regenerating line. The majority of the resulting F_1s had regeneration rates similar to those of the high regenerating parent, and thus the regeneration ability of the less responsive lines could be improved by crossing with high responding lines. Occasionally regeneration rates above that of the higher regenerating parent were observed but this was usually in low x low, or low x non-regenerating crosses. Analysis of variance of the data showed that both additive and dominant gene effects were significant. These findings are consistent with the results obtained from the genetic analysis of regeneration ability in other crops such as rice (Peng and Hodes, 1989; Taguchi-Shiobara *et al.,* 1997a) and barley (Komatsuda *et al.,* 1989), see Table 2.

In the study by Ono and Takahata (2000) significant maternal effects were observed only when 2 of the 7 genotypes were used as parents. By eliminating these two genotypes and using a 5 x 5 sub-diallel, maternal effects became non-significant. In this sub-diallel completely dominant (the higher regenerating lines) and completely recessive lines (a low and a non-regenerating line) were observed. Further analysis showed that the action of the dominant genes was positive for shoot regeneration. Similar results were found in regeneration from seed-derived callus of rice (Takeuchi, Abe and Sasahara, 1997; Taguchi-Shiobara *et al.,* 1997a). In contrast, a negative effect of dominant genes for regeneration was reported from leaf discs of tomato

(Frankenberger *et al.,* 1981); and from anther cultures (Quimino and Zapata, 1990) and seed-derived cell suspension cultures (Tsukahara *et al.,* 1995) of rice.

The genetic analysis of shoot regeneration in *B. oleracea* was first studied in 1974 by Buiatti *et al.,* in the form of an incomplete diallel. The conclusions from this work were that regeneration of shoots from leaf vein derived callus appeared to be controlled by additive genes. The data presented were incomplete, so it is unclear whether maternal effects were noted, but what again is clear is that regeneration appears to be an inherited trait. Hansen *et al.,* (1999) studied the genetic analysis of shoot regeneration from protoplasts in *B. oleracea*, by crossing a high and a low regenerating line and looking at the regeneration response in the F_2. The frequency distributions observed during short-term culture (*i.e.* the number of lines forming shoots 5 weeks after transfer of individual calli to regeneration medium) could be explained by two independent loci, with dominant genes controlling regeneration. In long-term culture (*i.e.* after 10-15 weeks) one additional dominant gene was found to be involved in regeneration *i.e.* three independent loci were responsible for regeneration. The finding that two or three genes control regeneration is consistent with other reports, see Table 2.

Species	Explant/Tissue	Genetic analysis	Reference
Cucumber	Leaf (seedling leaves)	2 dominant genes associated with shoot regeneration.	Nadolska-Orczyk and Malepszy, 1989
Dactylis glomerata (Orchard grass)	Leaf tissue	Contained two independent dominant genes associated with the production of somatic embryos.	Tar'an and Bowley, 1997
Melon	Leaf	Two dominant genes associated with shoot regeneration.	Molina and Nuez, 1996
Rice	Immature embryos Seed-derived calli	Both additive and dominant genes were associated with shoot regeneration (with additive genes being more important than dominant).	Peng and Hodes, 1989 Taguchi-Shiobra, 1997 a/b
Rice	Seed-derived calli	Shoot regeneration controlled by a single dominant gene.	Takeuchi, Abe and Sasahara, 1997
Rice	Seed-derived calli	Two independent genes, one dominant and one recessive controlled high shoot regeneration ability. Expression of either or both genes will result in high regeneration.	Zhang and Kazumi, 1998
Solanum phureja	Leaf disc	Recessive alleles in two loci were responsible for high shoot regeneration.	Taylor and Veilleux, 1992
Solanum chacoense	Leaf explants	Three unlinked genes associated with shoot regeneration.	Birhman *et al.*, 1994 Van Sint Jan *et al.*, 1996
Tomato	Leaf	Two dominant genes associated with shoot regeneration.	Koornneef *et al.*, 1987
Wheat	Immature embryos	One or few genes with major effects for callus growth were located on Chromosome 4B	Higgins and Mathias, 1987
Wheat	Immature embryos	Chromosomes 2B and 6D were critical for tissue culture response (*i.e.* callus growth and plantlet regeneration). Whereas chromosome 1D effects callus weight only. Chromosomes 1A and 3D were found to be critical but may carry genes with minor effects.	Ben Amer *et al.*, 1995
Wheat	Immature embryos	Three QTL controlling tissue culture responses located on chromosome 2B.	Ben Amer *et al.*, 1997

Table 2 Genetics associated with *in vitro* regeneration

Species	Explant/Tissue	Genetic analysis	Reference
Alfalfa	Cotyledons	Callus initiation was a single-locus-controlled trait showing complete dominance.	Hernandez-Fernandez and Christie, 1989
Alfalfa	Cotyledons	Callus proliferation showed additive genetic variation to be the major component of genetic variation (therefore potential for breeding in trait high).	Kielly and Bowley, 1997
Barley *(Hordeum vulgare)*	Immature embryos	Both additive and dominant genes were associated with shoot regeneration.	Komatsuda *et al.*, 1989
Barley	Immature embryos	Shd1 gene mapped to Chromosome 2. Shd1 accounted for 65% of total genetic variation for shoot regeneration.	Komatsuda *et al.*, 1993
Barley	Immature embryos	Contained two QTL's associated with callus production and four shoot regeneration genes.	Mano *et al.*, 1996
Barley	Anther	Anther-culture response: Three regions were associated with plant regeneration on chromosomes 2H and 3H.	Manninen, 2000
Barley	Immature embryos	QTLs for shoot differentiation associated with 1H, 2H and 3H. With the QTL on 3H having the largest effect.	Mano *et al.*, 2002
Brassica napus	Cotyledonary explants	Both additive and dominant genes were associated with shoot regeneration (with additive genes being more important than dominant).	Ono and Takahata, 2000
Brassica napus	Flower Buds	Microspore embryogenic ability is controlled by two loci with additive effects.	Zhang and Takahata, 2001
Brassica oleracea	Leaf veins	Callus growth and bud formation shown to be under additive control.	Buiatti *et al.*, 1974
Brassica oleracea	Leaf protoplast	Three independent loci associated with shoot regeneration.	Hansen *et al.*, 1999

1.4.2 Susceptibility to *Agrobacterium*

The ability to regenerate shoots is not the only pre-requisite for an efficient transformation system. *Agrobacterium* based transformation systems rely on plants being susceptible to *Agrobacterium* infection. If it is not possible to introduce T-DNA into the plant cell, the selected genotype cannot be transformed. As *Agrobacterium* is a natural plant pathogen, that infects wounded dicotyledonous plants *in vivo*, it is to be expected that a degree of resistance will have developed, in some species.

Wild-type *A. tumefaciens* causes crown gall disease, which is an important problem for nursery and field production of many stone fruit and nut crops (Kennedy and Alcorn 1980). Soil fumigation is an effective means of control but breeders are keen to introduce genetic resistance into their breeding lines to reduce the need for spraying. A number of studies have been carried out to look for resistance to crown gall formation (and therefore resistance or tolerance to *A. tumefaciens*). Work on species such as *Prunus* (Bliss *et al.,* 1999) and grapevine (Stover *et al.,* 1997) concluded that resistance to crown gall disease was genotype dependent and under genetic control. In soybean, Mauro *et al.,* (1995) proposed a one to two gene theory for susceptibility to *Agrobacterium.*

Another approach towards investigating *Agrobacterium* susceptibility (to improve the efficiency of transformation) has been to screen a range of *Agrobacterium* strains against plant genotypes of interest. This enables the identification of the most susceptible plant genotypes and most virulent *Agrobacterium* strains (generally nopaline strains) Lowe *et al.*, (1993). These approaches failed to predict transformation success based on susceptibility to crown gall formation.

In *Arabidopsis*, Nam *et al.,* (1997) concluded that differences in susceptibility to crown gall disease between *Arabidopsis* ecotypes might result from a deficiency in

T-DNA integration. They also found in one ecotype that not only was susceptibility inherited, but it also segregated as a single major contributing locus (however, the possibility of additional segregating loci contributing to tumorigenesis, could not be ruled out). Nam *et al.,* (1999) identified several T-DNA-tagged mutants of *Arabidopsis* that were highly recalcitrant to *Agrobacterium* root transformation. These mutants were named *rat* mutants (resistant to *Agrobacterium* transformation). In most of these mutants *Agrobacterium* transformation is blocked at an early step, either during bacterial attachment to the plant cell or before T-DNA nuclear import. In some of the mutants, however, the T-DNA integration step is most likely blocked. Mysore *et al.,* (2000) showed that in *rat5* a histone H2A gene is disrupted. Complementation analysis and *RAT5* over expression indicated that H2A plays a role in *Agrobacterium* transformation. The T-DNA integration stage of transformation is blocked in the *rat5* mutant. This suggests that histone H2A (RAT5) plays an important role in illegitimate recombination of T-DNA into the plant genome.

In summary, genotype susceptibility to *Agrobacterium* may well affect transformation efficiency. The potential 'block' to *Agrobacterium* could arise at a number of stages; attraction of *Agrobacterium* to wounded cells, *Agrobacterium* attachment to plant cell, or transfer of T-DNA into the plant cell / nucleus / chromosome. Variations in any of these, individually and in combination, would be expected to influence transformation efficiency.

1.4.3 Selection

During the co-cultivation period with A. *tumefaciens* only a small proportion of plant cells will be transformed. For this reason a selectable marker gene is included within the T-DNA to confer an advantage to transformed cells, over non-transformed cells. A list of the most commonly used selectable marker genes is given in Table 3.

To date the most commonly used selectable marker is the neomycin phosphotransferase gene (*nptII*) which confers resistance to aminoglycoside antibiotics such as kanamycin.

The level or concentration of the selective agent (e.g. kanamycin) used is variable depending on the plant species, and even genotype within a species, to be transformed. When developing a transformation system 'kill curves' are used, where ranges of selective agent concentrations are tested to determine the optimum level for selection for a given genotype. If the concentrations of the selective substances in the culture media are not high enough the number of non-transformed shoots (escapes) developing will often be large, and this will considerably reduce the transformation efficiency. If the concentrations used are too high, non-transformed cells are likely to become necrotic and adversely effect the early development of transformed cells. At the correct level, kanamycin generally causes chlorosis rather than necrosis of untransformed cells. Thus, non-transformed cells are possibly able to provide some developmental support to the early developing transformed cells. Any shoots that develop from non-transformed cells will be chlorotic (white) and therefore easy to distinguish from transformed green shoots. The optimal level of the selective agent required often varies with plant species and genotype, due to some genotypes having an inherent background resistance to some selective agents.

Table 3 Selectable marker genes

Marker Gene	Enzyme Encoded	Resistance conferred antibiotics
nptII	neomycin phosphotransferase	kanamycin neomycin G418 paromomycin
hpt or *aph IV*	hygromycin phosphotransferase	hygromycin
dhfr	dihydrofolate reductase	methotrexate
Herbicides		
bar	phosphinothricin acetyltransferase	phosphinothricin
aro A	5-enolpyruvylshikimate-3-phosphate synthase	glyphosate
modified *als* genes	acetohydroxyacid synthase (or acetolactate synthase)	chlorsulfuron imidazolanones

'Positive selection' systems can also be used to give an advantage to transformed cells. For example, the majority of plants are unable to metabolise either mannose or mannose-6-phosphate, but the introduction of a gene for phosphomannose isomerase that converts mannose-6-phosphate into fructose-6-phosphate facilitates growth of transformed cells on culture media containing mannose. This positive selection system has been used successfully in *B. napus* transformation (Bojsen *et al.*, 1994).

Transformed plants produced under positive selection also have the advantage (from an international regulatory perspective) of not containing antibiotic resistance genes in the end product. Concerns over selectable marker (e.g. antibiotic resistance) genes remaining in transformed products have led to the development of so called 'clean-gene' technology. Selectable marker genes are introduced into the host plant using a different plasmid from the plasmid containing the genes of interest or by introducing two T-DNA into the same plasmid. The removal of undesirable antibiotic

resistance genes as with *A. rhizogenes* systems is via genetic recombination and selection in subsequent sexual generations.

1.5 Project aims and objectives

The aim of this research is to understand the genetic control of the critical stages during *A. tumefaciens* mediated transformation of *Brassica*. This work follows on from preliminary studies carried out to identify variables associated with transformation in horticultural brassicas (data presented in Appendix A). Genetic variation for shoot regeneration and tissue sensitivity to *Agrobacterium* infection were further investigated using a doubled haploid mapping population. Inheritance patterns and genetic loci for these traits were identified, and their interaction and effect on transformation efficiencies analysed and discussed. The genetic control of background resistance to antibiotics was not further investigated in the doubled haploid population, for reasons outlined in Appendix A, section A.5, page xiv.

The ability to identify what makes some genotypes amenable to transformation while others remain recalcitrant will be of potential benefit to transformation programmes. The findings from this research will enable researchers to move away from the traditional empirical approach towards the use of transformation methods, to a more scientific approach. Understanding the genetic control of 'transformation' traits should lead to the identification of genetic loci and genetic markers that will facilitate the selection of amenable breeding genotypes for use in GM breeding programmes. The introduction of favourable traits / genes to improve the transformation efficiency of recalcitrant lines may ultimately also be possible.

Chapter 2 Materials and Methods

2.1 Plant material and genetic map

This study uses a reference doubled haploid (DH) population derived from a cross between *Brassica oleracea* ssp *alboglabra* (A12DHd) and *B. oleracea* ssp *italica* (Green Duke GDDH33). The population was originally produced to create a RFLP (restriction fragment length polymorphism) map of *B. oleracea,* by Bohuon *et al.,* (1996) at the John Innes Centre, Norwich, UK and public access to this material can now be obtained via G. King of Horticultural Research International, Wellesbourne, UK. This population and the RFLP linkage map were subsequently used to develop an integrated map with additional AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeat) loci incorporated (Sebastian *et al.,* 2000). The integrated map of Sebastian *et al.* (2000) was used in this study.

Seed material of 60 doubled haploid (DH) lines plus the two parental lines A12DHd and GDDH33, were made available from the John Innes Centre, Norwich, UK and Horticultural Research International, Wellesbourne, UK for use in this study.

2.1.1 Generation and maintenance of plant material

DH lines were maintained in a lit glasshouse (with a 16-hour photoperiod, +18/12°C day/night) and self-pollinated, in order to generate the seed numbers required for use in this study. Plants were covered with clear, perforated 'bread-bags' (Cryovac (UK) Ltd) as soon as they came into flower to prevent cross-pollination. For those lines that were self-compatible periodic shaking of the 'bread-bag' was enough to facilitate pollination. A number of the DH lines were self-incompatible (SI) and in most cases SI could be overridden by either hand pollination or by use of a saline pre-treatment on

open flowers. Hand pollination involved opening up a closed bud and brushing fresh pollen onto the newly exposed stigma. The saline pre-treatment was carried out by applying a 5 % NaCl, 0.1 % Tween-20 solution onto the stigma of a newly opened flower with a fine artist's paintbrush. After 2 hours, fresh pollen was then transferred to the stigma of the open flower (Carafa and Carratu, 1997). Pods were allowed to develop on the plant until fully swollen and were harvested when they had dried and turned brown. Harvested pods were threshed when dry, and seed stored in the John Innes Centre seed store (+ 1.5°C, 7-10 relative humidity).

2.1.2 Generation of the F_1 population (12 x 12 Diallel)

A subset of 12 DH lines was selected for use in a diallel-crossing programme (the selection criteria for the diallel is described in Chapter 3, section 3.3.1). Each DH line was crossed with every other individual line both as a maternal and paternal parent (reciprocal crosses) and individual lines were also self-pollinated. For the 12 DH parental lines, therefore, there were 12^2 potential crosses.

A 12 x 12 diallel is a substantial undertaking and great care was taken to avoid any pollen contamination, which would spoil the validity of this population. Prior to pollination, hands and tweezers (Biology Grade Type 5 (T083) Dumont, Aldermaston, UK) were washed in 70 % ethanol. A raceme of buds was selected, when plants were used as the maternal donor, and buds too small for pollination were removed along with any signs of lateral buds and the meristem – this would prevent any subsequent flowering. Buds were opened and the anthers removed before pollen was taken from the paternal donor and brushed onto the stigma of the maternal plant. When all buds had been pollinated a 'bread-bag' was placed over the pollinated raceme and tied with a tag denoting the two parents of the cross. Care was taken to make sure the bag did not rest on the stigmas, as this often resulted in failure of the cross. When a line was used

as the paternal donor, fresh pollen was used. Periodically, excess flowers were removed from the plants to ensure fresh pollen was available (rather than using pollen from older flowers) and also to reduce the amount of pollen that could lead to cross-contamination. Pods were harvested when they had started to dry on the plant and lines were threshed by hand. Once harvested each F_1 cross was given an individual code number (Table 4) for future reference.

Table 4 The 12 x 12 diallel table

The P number refers to the individual code name for each F_1 cross.

Male / Female	5070	3070	5047	5117	4052	6024	4030	2072	5118	2069	1027	1002
5070	**5070**	P44	P86	P87	P46	P90	P45	P43	P88	P42	P47	P85
3070	P134	**3070**	P133	P124	P135	P125	P98	P83	P99	P132	P130	P131
5047	P82	P11	**5047**	P13	P95	P15	P12	P10	P14	P59	P9	P81
5117	P50	P49	P103	**5117**	P89	P52	P74	P104	P51	P48	P47	P102
4052	P39	P38	P34	P40	**4052**	P41	P71	P37	P117	P36	P35	P84
6024	P55	P54	P114	P56	P77	**6024**	P76	P53	P94	P75	P79	P93
4030	P101	P32	P67	P33	P123	P70	**4030**	P68	P69	P111	P57	P100
2072	P120	P29	P108	P109	P30	P106	P66	**2072**	P31	P28	P65	P121
5118		P127		P128	P92	P105	P91		**5118**	P126	P113	
2069	P26	P24	P21	P27	P112	P122	P25	P23	P129	**2069**	P22	P64
1027	P19	P17	P80	P20	P18	P63	P107	P61	P116	P16	**1027**	P60
1002	P6	P4	P1	P7	P5	P8	P110	P3	P115	P119	P2	**1002**

The absence of a P number denotes an unsuccessful cross where F_1 seed were not obtained. Self-crosses are shown in red.

2.1.3 Generation of the BC and F_2 populations

Each F_1 was backcrossed reciprocally to both parental lines. Cross-pollination techniques were as described for the diallel. For the generation of the F_2 population, up to six F_1 seed from each of the successful crosses were sown out, and maintained in a lit glasshouse (16-hour photoperiod, +18/12°C day/night). After 8 to 10 weeks the plants were staked and bagged. Once in flower the bags were shaken daily to aid self-

pollination. All plants produced self-seed without the need for hand-pollination. Plants were allowed to set seed, and when pods started to dry the stems of the plants were broken, and pods were allowed to further dry. F_2 seed numbers were very high (1000 plus in most cases).

2.2 Production of sterile seedlings *in vitro*

All the experimental trials described within this thesis were carried out using sterile seedling material germinated *in vitro*.

2.2.1 Surface sterilisation of seed

Seeds were surface sterilised by rinsing in 100 % ethanol for 2 minutes, followed by sterilisation in 15 % sodium hypochlorite (BDH) for 15 minutes. To remove excess sodium hypochlorite, seeds were rinsed three times for 10 minutes in sterile distilled water.

2.2.2 Germination of seed

Seeds were germinated on MS (Murashige and Skoog, 1962) basal medium (Table 5), at a density of 15 seed per 90 mm petri dish (Bibby Sterilin Ltd. 101R20) for regeneration or transformation trials, or 25 seed per Phytatray® (Sigma P-5929) for *Agrobacterium* susceptibility trials. Petri dishes / phytatrays were transferred to a 10°C cold room overnight before being transferred to a 23°C culture room under 16 hour daylength of 70 μmol m^{-2} sec^{-1}

2.3 *In vitro* shoot regeneration

Both cotyledon and hypocotyl explants were excised from 4-day-old seedlings, and maintained on regeneration medium (Table 5). Cotyledonary explants were excised with approximately 2-5 mm of petiole attached (ensuring no apical meristem tissue was attached) and hypocotyl explants were taken of approximately 1 cm length segments. Hypocotyl explants were laid onto the surface of the regeneration medium and cotyledonary petioles were embedded into the medium, ensuring the cotyledonary lamellae remained above the medium. Deeper 90 mm petri dishes were used for regeneration (Falcon 3003). Explants were maintained on regeneration medium, in a 23°C culture room under 16 hour daylength of 70μmol m^{-2} sec^{-1}. Explants were sub-cultured onto fresh regeneration medium after 23 days in culture.

2.4 Infection with wild-type *Agrobacterium tumefaciens*

Three wild type strains of *A.tumefaciens* were used in this study; two nopaline strains C58 and T37 (Sciaky *et al.*, 1978), and the octopine strain Ach5 (Ooms *et al.*, 1981). *A. tumefaciens* stains C58 and T37 were obtained from N. McKenzie (John Innes Centre, Norwich, UK) and Ach5 from M. Christey (Crop and Food Research, Christchurch, New Zealand).

2.4.1 Growth of *Agrobacterium*

Prior to plant inoculations, all *A. tumefaciens* strains were streaked out onto LB solid medium containing selection (25 μg/ml naladixic acid). Selection was not required for T37. Plates were incubated at 28°C for 48 hours, after which a single colony of each strain was transferred to 10 ml of Minimal A liquid medium containing

the appropriate level of selection and transferred to a 28°C shaker for 48 hours. An aliquot of 50 µl of resulting bacterial suspension was then transferred to 10 ml of Minimal A liquid medium containing no selection and grown over night in a 28°C shaker. To ensure all bacterial suspensions used for explant inoculation were of the same concentration, overnight suspensions of $O.D_{650} = 0.1$ were used for inoculations (dilutions made using Minimal A liquid medium). The preparation of LB and Minimal A medium is given in section 2.10.

2.4.2 Inoculation with wild-type *Agrobacterium*

Fine needles (Terumo® 0.5 x 25 mm) dipped into bacterial suspensions of $O.D_{650nm} = 0.1$ were used to inoculate five-day-old seedlings just below the meristem. To ease handling of explants roots were removed prior to infection and seedlings were laid onto empty petri dishes in preparation for the inoculation stage. To ensure infection, 1.2 µl of bacterial suspension was subsequently pipetted onto the injection site. Five seedlings were established and placed into Phytatrays® (Sigma P-5929) containing MS basal medium (Table 5) ensuring the injection site was above the medium. Explants were transferred after 3 days to fresh medium (MS basal medium, supplemented with 500 mg/l carbenicillin, to eliminate *Agrobacterium* overgrowth). Controls were also established, where seedlings were inoculated with Minimal A liquid medium containing no *A. tumefaciens*. All explants were scored for the presence or absence of crown galls 10, 20, 30, 40 and 50 days after infection.

Table 5 Media composition

Deviations from these three basic media are given within the text.

Medium Components	MS Basal	Regeneration / Co-Cultivation	Gambourgs B5
MS plant salt base Duchefa – M0222	4.4 g/l	4.4 g/l	n/a
Gamborg's B5 salts Duchefa – G0209	n/a	n/a	3.1 g/l
Sucrose	30 g/l	30 g/l	10 g/l
Phytagar® GIBCO – 10675-023	8 g/l	8 g/l	8 g/l
pH	5.7	5.7	5.7
BAP 6-Benzylaminopurine Sigma – B-9395	n/a	2 mg/l	n/a
Filter sterilised (added post autoclaving)			
myo-Inositol Sigma – I-3011	100 mg/l	100 mg/l	n/a
Thiamine-HCL Sigma – T-3902	10 mg/l	10 mg/l	n/a
Pyridoxine Sigma – P-8666	1 mg/l	1 mg/l	n/a
Nicotinic acid Sigma – N-0765	1 mg/l	1 mg/l	n/a

2.5 Plant transformation

2.5.1 Transgene construct and *Agrobacterium* strains

Two *A. tumefaciens* strains LBA 4404 (Hoekema *et al.*, 1983) and EHA 101 (Hood *et al.*, 1986), were used for the transformations outlined in Chapter 5. Both strains harboured the plasmid p25GI, see Figure 2. The T-DNA of the plasmid contains

the reporter gene β-glucuronidase (gus) which contains an intron, and neomycin phosphotransfertase (*nptII*) as the selectable marker gene. A 35S promoter, from the Cauliflower mosaic virus, is used to drive both genes.

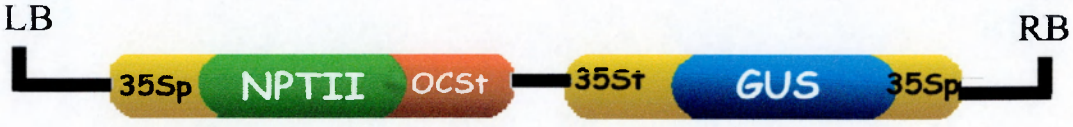


Figure 2 **Diagrammatic representation of the p25GI construct**
Figure courtesy of N. Al'Kaff, John Innes Centre, U.K.

2.5.2 Transformation procedure

The transformation protocol was developed from that of Moloney *et al.*, 1989. Cotyledonary petioles excised from 4-day-old seedlings were dipped into an overnight suspension of *Agrobacterium* (section 2.4.1). Explants were transferred to co-cultivation medium (Table 5). Cultures were maintained in growth rooms at 23°C with 16 hour daylength, and under scattered light of 40μmol m^{-2} sec^{-1} for 72 hours. After 72 hours explants were transferred to selection medium, consisting of the co-cultivation medium supplemented with 500 mg/l carbenicillin (to eliminate bacterial growth) and 25 mg/l kanamycin as the selection agent. Care was taken to make sure the cotyledonary lamellae remained above the medium.

Green shoots that regenerated were excised and transferred to 100 ml Sterilin jars containing Gamborgs B5 medium (Gamborg *et al.*, 1968) (Table 5) supplemented with 500 mg/l carbenicillin and 25 mg/l kanamycin. Shoots were maintained on this medium until roots developed. Plantlets were then transferred to sterile peat pots (Jiffy No.7) to

allow further root development, before being transferred to the glasshouse. Confirmation of transformation was via molecular and histochemical analysis.

2.6 GUS histochemical analysis of putative transgenic plants

Leaf tissue was taken from putative transgenic shoots *in vitro*, and transferred to 48 well plates (Fisher Scientific, TKT-190 090H). Leaf tissue was immersed in 200 µl X-GLUC solution (section 2.10) and plates vacuum infiltrated for 2 minutes (to ensure X-GLUC penetration of the leaf tissue). Plates were wrapped in foil and incubated at 37°C overnight. The presence of blue colouration to the leaf tissue is indicative of β-glucuronidase enzyme activity *i.e.* GUS expression (Jefferson *et al.*, 1987).

2.7 Molecular analysis of putative transgenic plants

2.7.1 Quick preparation of DNA for PCR analysis

Leaf tissue (of ~1.5 cm^2) was taken from putative transgenic shoots and transferred to 1.5 ml tubes on ice. The tissue was ground in the presence of 50 mg of sand and 200 µl CTAB (1) buffer (section 2.10) and incubated at 65°C for 10 minutes. In a fume hood 200 µl chloroform was added, the tubes mixed by vortexing and centrifuged at 13, 000 rpm for 2 minutes. The supernatant was transferred to a clean 1.5 ml tube and mixed with 200 µl of 100 % Propan-2-ol, and left at room temperature for 15 minutes, before being centrifuged at 13, 000 rpm for 15 minutes. The pellet was washed in 70 % ethanol, and then air-dried. Pellets were resuspended in 50 µl of H_20 and left overnight at room temperature. Samples were stored at 4°C.

2.7.2 Polymerase Chain Reaction (PCR)

PCR analysis was carried out to confirm the presence of *nptII* and *gus* genes in putative transgenics. PCR reactions were carried out in 0.5 ml tubes with a reaction volume of 50 µl containing;

5 µl Buffer (10 X PCR buffer; Amersham Pharmacia Biotech Inc.)

2 µl dNTPs (Promega)

2 µl of Primer A (*gus* or *nptII* from a 5 mM stock)‡

2 µl of Primer B (*gus* or *nptII* from a 5 mM stock) ‡

0.5 µl Taq Polymerase (Amershem Pharmacia Biotech Inc.)

33.5 µl Sterile distilled water

5 µl of extracted and resuspended plant DNA

‡ **Primer Sequences:** Supplied by SigmaGenosys.

nptII	5' GAG GCT ATT CGG CTA TGA CTG G 3'
	5' ATC GGG AGC GGC GAT ACC GTA 3'
gus	5' GGT GGG AAA GCG CGT TAC AAG 3'
	5' GTT TAC GCG TTG CTT CCG CCA 3'

Both *nptII* and *gus* PCR were carried out using the following programme on a Hybaid TouchDown PCR machine.

- Hot start protocol — 94 °C 5 minutes

- Denaturation temperature — 94 °C 30 seconds
  Annealing temperature — 60 °C 30 seconds
  Extension temperature — 72 °C 1 minute 30 seconds
  Number of cycles — 35

- Finish auto extension — 72 °C 10 minutes
- End holding temperature — 4 °C

A 5 µl aliquot of loading buffer (bromophenol blue (Sigma)) was added to each tube after PCR, and 20 µl of the PCR product was analysed, by electrophoresis, on a 1 % agarose gel containing ethidium bromide (0.5 µg/ml). The presence of the *nptII* and *gus* genes was confirmed by bands of fragment size 0.7 and 1.0 kb respectively.

2.8 Southern analysis

2.8.1 Plant DNA Extraction

Approximately 0.5 g of freeze dried leaf tissue was weighed and dispensed into 30 ml milling tubes containing 2 ball bearings. The tissue was milled for 20 minutes in a Spex 8000 milling machine to produce fine powder. The powder was transferred to a 50 ml polypropylene centrifuge tube containing 30 ml of Extraction Buffer (section 2.10), and incubated at 65°C for 45 minutes. A 10 ml volume of 5M potassium acetate was added, the tubes mixed by vortexing and centrifuged for 10 minutes at 3,000 rpm at 4°C. The whole mixture was filtered through Miracloth (CalBiochem.) and the flow through divided equally between two 50 ml polypropylene centrifuge tubes. A 2X

volume of 100% ethanol was added to each tube, mixed gently and centrifuged at room temperature (R.T.) for 10 minutes at 3,000 rpm. The pellets were washed in a 10 ml volume of 80 % ethanol, and further centrifuged for 3 minutes at 3,000 rpm (R.T.). Pellets were air-dried for 15 minutes. A 2 ml volume of preheated (65°C) TE (100mM Tris-HCL, pH7.4; 10 mM EDTA) buffer pH 7.5 containing 10 µg/ml RNase A (Sigma) was added to each of the two tubes, and the whole recombined into one 50 ml polypropylene centrifuge tube. Tubes were incubated at 65°C for 30 minutes, until the pellets resuspended. An equal volume of CTAB(2) Buffer (see section 2.10) at 65°C was added, the tubes mixed and incubated at 65°C for 15 minutes. An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) was added, the tubes mixed by vortexing, shaken for a further 15 minutes and centrifuged for 15 minutes at 3,000 rpm (R.T.). The resulting aqueous upper layer containing the extracted DNA was removed to a fresh 50 ml tube. An equal volume of chloroform was added, the tubes mixed by vortexing and centrifuged for 5 minutes at 3,000 rpm (R.T.). The resulting supernatant was transferred to a new 50 ml tube. An equal volume of Propan-2-ol was added to precipitate out the DNA, the tubes were mixed by inverting several times and left over night. Tubes were centrifuged for 10 minutes at 3,000 rpm (R.T.); the pellet was washed in a 2 ml volume of 80 % ethanol, and transferred to a 1.5 ml tube and washed again with 80 % ethanol. The purified DNA pellets were air-dried and resuspended in 250 µl of 1 x TE buffer and stored at –20°C.

2.8.2 Quantification of DNA and determination of DNA quality

A 1 µl volume of extracted DNA was run out on a 1 % agarose test gel, alongside DNA samples of known concentration ([µg/ml] = A_{260} x 50 x dilution factor). DNA was quantified by eye, by comparing with those samples of known concentration [20 µg]. DNA samples that showed signs of degradation were not subsequently used.

2.8.3 Restriction enzyme digest and separation of DNA fragments by agarose gel electrophoresis

Each DNA sample (20 µg) was digested with *Eco* R1 restriction endonuclease (Roche), using 2 units of enzyme per 1 µg of DNA and incubated overnight at 37°C. To 30 µl of digest, 3 µl of loading buffer (containing RNase) was added. Electrophoresis of the DNA digests was carried out on a 20 cm x 20 cm, 1 X TAE, 1.0 % agarose gel at 1.5 V/cm for approximately 20 hours (or until the bromophenol blue dye front had moved approximately 12 cm from the wells). The gel was then stained for 30 minutes in 500 ml of 0.5 µg/ml ethidium bromide in 1 X TAE buffer, and visualised on a gel imagining system (BioDoc-It™ Transilluminator System, UVP) to check digestion.

2.8.4 Capillary Blotting

After electrophoresis and staining with ethidium bromide, the gel was trimmed to 20 x 12 cm (just below the bromophenol blue dye front). The DNA was transferred onto a 20 x 12 cm Hybond N+ membrane (Amersham) by capillary blotting overnight using 0.4 M NaOH as the transfer solution. The filter was rinsed with 2 x SSC (300 mM NaCl, 30 mM tri-sodium citrate, pH 7.0) wrapped in Saran Wrap® (Genetic Instrumentations Ltd.) and stored at 4°C.

2.8.5 Southern hybridisation, with pW233

2.8.5.1 Pre-hybridisation

Filters were pre-hybridised in glass tubes (Hybond – HBOVBM) containing 30 ml of Hybridisation buffer (section 2.10) at 65°C, in a rotary oven for 2 hours.

2.8.5.2 Labelling of probe

The RFLP probe pW233 (Sharpe *et al.*, 1995) was supplied by M. Trick (John Innes Centre, Norwich, U.K). A 4 µl volume of this probe was added to 8 µl of sterile distilled water in a screw topped 1.5 ml tube and boiled for 5 minutes to denature, then immediately cooled on ice for 5 minutes before being spun down. Using the Random Primer DNA labelling system (Invitogen: 18187-013), 3 µl of nucleotide mix (dATP, dTTP, dGTP), 7.5 µl random primer mix and 0.5 µl klenow was added to the probe. A 2 µl volume of ^{32} P dCTP was added to the mixture and left to incubate for 2 hours at room temperature.

2.8.5.3 Hybridisation

Hybridisation tubes were decanted to leave 4 mls of buffer in each of the tubes. The probe was boiled for 5 minutes, then cooled on ice for a further 5 minutes before being added to the hybridisation tubes. Hybridisation was carried out at 65°C in a rotary oven, and left over night.

2.8.5.4 Washes

Five ml of Wash A (2 x SSC, 1 % SDS) was added to the hybridisation tubes to rinse the filters quickly and then the probe was discarded down a designated radioactive sink. The filters were then washed for 2 x 10 minutes in 30 – 50 ml of Wash A in the oven at 65°C. Filters were transferred to a large plastic box and washed for a further 10 minutes with Wash B (2 x SSC, 0.1 % SDS). Washing with Wash B was repeated until just ~ 10 to 15 counts per second remained bound to the filter.

2.8.5.5 Exposure

Filters were wrapped in Saran Wrap® and exposed to X-ray film (Kodak X-OMAT AR X-ray) (at -70 °C) for 1 to 14 days depending on the filter counts per second. X-ray film was developed using a X-O GRAPH X2 developer (Fuji). Alternatively, filters were put down with a phosphor-imaging plate (BAS-IP MS 2040, Fuji) and left at R.T overnight, before being read on an imaging machine (Thyphoon 8600, variable mode imager).

2.9 Quantitative trait loci (QTL) analysis

QTL were identified using the computer programme MAPQTL® version 3.0 (Van Ooijen and Maliepaard, 1996) and located by two point analysis using a LOD threshold of 3.0 (Van Ooijen, 1999). The mapping data used for this programme were made available as an Excel spreadsheet from the Brassica Database (http://jic-bioinfo.bbsrc.ac.uk/BrassicaDB/BrassicaDB/Bo_map_data.xls) and transferred to JOINMAP® (Stam and Van Ooijen, 1995) for use in MAPQTL® version 3.0.

Single marker analysis was performed using two computer programmes RFLPQB and RFLPRandom (R. Mithen, Nottingham University and P. Fretter, John Innes Centre, Norwich, UK, pers. comm.) to identify RFLP markers that were putatively linked to QTL. Analysis of variance (ANOVA) was used to compare the trait levels of the DH population at each of the RFLP loci identified. Threshold values of significance were calculated following the approach described by Churchill and Doerge (1994) and applied by Ray *et al.,* (1996). This process involved reassigning the phenotypic data at random, for the DH population, and determining the maximum t value. This procedure was repeated 10,000 times and the maximum t value for each cycle ranked in increasing order of magnitude. The 9999^{th}, 9990^{th}, 9900^{th} and 9500^{th} values provided the t values for the 0.0001, 0.001, 0.01 and 0.05 levels of significance. In this procedure, threshold values are re-calculated for every phenotypic data set. Values obtained above these thresholds indicate that the trait is significantly associated to a particular marker, and that this occurrence is not purely by chance.

2.10 Reagents

Sigma-Aldrich Co.Ltd. (Poole, Dorset, UK), BDH (Merck Ltd, Lutterworth, Leicestershire, UK) or Duchefa Biochemie BV (distributed by Melfords Laboritories Ltd. Ipswich, UK) supplied all chemicals used in these studies unless otherwise stated.

LB Medium

Yeast Extract	5 g/l
NaCl	10 g/l
Tryptone	10 g/l
Bactoagar (Difco)	15 g/l

Minimal A Medium: To make 1L

50 ml Minimal A Salts
50 ml Minimal A Buffer
10 ml 20% Sucrose
1 ml 1M $MgSO_4$
890 ml SDW

When making Minimal A medium all components were autoclaved separately (with the exception of $MgSO_4$, which is unstable at high temperatures, so was filter sterilised), before combining.

20X Minimal A Salts

$(NH4)_2SO_4$ 20 g/l
Sodium Citrate 10 g/l

20X Minimal A Buffer

K_2HPO_4	274 g/l
KH_2PO_4	90 g/l

X-GLUC solution: For histochemical GUS analysis

To make 100 mls.

Dissolve 0.2 mg/ml X-GLUC in a small volume of dimethyl formamide (200 µl).
Add 50 mM $NaPO_4$, pH 7.0 (up to 100 ml).
Add two drops of Triton-100.
(Store foil wrapped at 4°C).
X-GLUC was supplied by Melfords (MB1121) as 5-bromo-4-chloro-3-indolyl β-D-glucuronide.

Hybridisation Buffer (for two tubes).

50 ml Sterile distilled water

18.75 ml 20 x SSPE

3.75 ml 50 x Denhardts (filtered)

3.75 ml 10 % SDS (filtered)

Hybridisation buffer was pre-heated to 65°C and 75 µl of freshly boiled 5 mg/ml Herrings testes DNA (Sigma D-6898) was added prior to adding to the hybridisation tubes.

50 x Denhardts Solution

5 g Ficoll (Type 400)

5 g Polyvinylpyrrolidone (360)

5 g BSA (Fraction V)

Make up to 500 ml with sterile distilled water.

Filter and store in aliquots at –20°C.

20 x SSPE

0.2 M Phosphate buffer (below)

3.6 M NaCl

0.02 M EDTA

Autoclave before use.

20 x SSC

3 M NaCl

0.3 M Sodium Citrate

1M Sodium Phosphate Buffer, pH 7.2

684 ml 1M Na_2HPO_4

316 ml 1M NaH_2PO_4

CTAB (1) Buffer

0.8 M NaCl

0.22 M TRIS-HCL pH 8.0

0.14 M Sorbitol

0.022 M EDTA

1 % n-laurylsarcosine

0.8 % CTAB (hexadecyltrimethylammoniumbromide)

Extraction Buffer	**CTAB (2) Buffer**
100 mM Tris-HCL	200 mM Tris-HCL
50 mM EDTA	50 mM EDTA
500 mM NaCl	2 M NaCl
1.25 % SDS	2 % CTAB (hexadecyltrimethylammoniumbromide)
8.3 mM NaOH	

Chapter 3 Investigating the genetic control of *in vitro* shoot and root regeneration from seedling explants of *Brassica oleracea*

3.1 Introduction

The introduction of foreign DNA into a plant genome from which subsequent regeneration of a complete plant is not possible would be a futile and costly exercise. Therefore, the ability to regenerate shoots (and subsequently roots) is an important pre-requisite for any transformation system. In Chapter 1, variation for shoot regeneration, both between and within the *Brassica* species, was discussed and included an assessment of evidence that shoot regeneration is under genetic control. This Chapter looks at the genetic control of *in vitro* shoot and root regeneration from two seedling tissue types (cotyledonary petiole and hypocotyl segments) within the A12DHd / GDDH33 doubled haploid (DH) mapping population of *B. oleracea*. The inheritance and genetic control of *in vitro* regeneration is further investigated in subsequent generations derived from this mapping population.

The populations detailed below were screened for both shoot and root regeneration potential from the two tissue types.

1. Fifty-five DH lines from the *B. oleracea* mapping population, along with the two parental lines A12DHd and GDDH33 (detailed in section 2.1).
2. The DH parents and the F_1 population generated in the 12 x 12 diallel programme (detailed in section 2.1.2).
3. Backcrossed and F_2 populations derived from a subset of F_1 lines from the diallel programme.

3.2 Screening of the DH mapping population for shoot (and root) regeneration

3.2.1 Method

3.2.1.1 Plant material and genetic map

Fifty five DH lines from the *B. oleracea* mapping population and the two parental lines, A12DHd and GD33DHd were screened for shoot and root regeneration from both cotyledonary petioles and hypocotyl segments. The genetic map of *B. oleracea* described by Sebastian *et al.,* (2000) was used to determine quantitative trait loci (QTL) associated with these traits.

3.2.1.2 Experimental procedure

The experimental procedure for shoot and root regeneration is outlined in detail in section 2.3. In total, 100 cotyledons (10 plates of 10 cotyledons) and 50 hypocotyls (5 plates of 10 hypocotyls) were established for each of the 55 DH lines and the two parental lines. Due to the size of this trial the experiment was set up over 5 weeks, with 20 cotyledon and 10 hypocotyl explants established for each of the genotypes each week. Statistical analysis confirmed there was no significant difference in regeneration response between trials set up over the 5 weeks, and therefore these could be considered as replicates. Explants were maintained on regeneration medium containing 2 mg/l BAP (as previously described) in a 23^0C culture room under 16 hour daylength of 40μmol m^{-2} sec^{-1} and scored after 16, 23 and 44 days for the presence or absence of regenerating shoots and roots. Explants were sub-cultured onto fresh regeneration medium after 23 days in culture.

3.2.1.3 Statistical procedures

Scoring for the presence or absence of a trait is a binomial score (0 or 1). Therefore, data are presented as counts (or frequencies). If all explants for a given genotype failed to regenerate shoots the score of 0 was given, if all explants regenerated shoots a score of 1.0 was given. If half the explants regenerated shoots a score of 0.5 was given (*i.e.* the number of explants regenerating shoots (*each having a score of 1*) / total number of explants established). The proportions of explants regenerating shoots or roots were analysed using generalised linear models (Abeyasekera and Stern, 2001) with logit link and binomial error in the software package Genstat version 5.0®.

3.2.2 Results and Discussion

Explants were scored after 16, 23 and 44 days in culture. Shoot regeneration from all responding lines appeared well before 44 days, and those that failed to regenerate shoots did not show any regeneration response if cultured for longer than 44 days. Therefore, 44 days (6 weeks in culture) was chosen as the final score date. Only the results from the 44 day score are presented here. It was apparent that some genotypes regenerated shoots at a rate faster than others, and those genotypes tended to be the high regenerating DH lines that produced multiple shoots. Due to the size limitation of the population screened, and the proportion of these genotypes that did regenerate shoots, comparing the speed of regeneration between genotypes of this population was not practicable.

The experiment was established primarily to investigate shoot regeneration, with culture conditions optimised for shooting. However, as rooting was such an apparent variable, this trait was also scored. It should be noted that the ability to regenerate adventitious roots, from explants *in vitro*, may or may not be related to the ability of

regenerated shoots to root *in vitro*. The scoring of adventitious rooting from explants was carried out to see if any antagonistic relationship exists between shooting and rooting, *i.e.* does the regeneration of roots from explants inhibit the regeneration of shoots?

3.2.2.1 Comparison of shoot regeneration in the two parental lines

The parental lines, GDDH33 and A12DHd, regenerated shoots in culture from both cotyledonary petioles and hypocotyl explants. The frequency of shoot regeneration from cotyledonary petioles was higher in A12DHd (0.68) than GDDH33 (0.15). Similarly, shoot regeneration from hypocotyl explants was higher in A12DHd (0.74) than GDDH33 (0.35). Although both genotypes regenerated shoots *in vitro*, the mode of regeneration for the two genotypes was different. GDDH33 regenerated shoots through a distinct callus phase from both cotyledon and hypocotyl explants whilst A12DHd regenerated shoots directly from the cut surface without obvious callus formation (see Figure 3).

A12DHd showed signs of blackening at the cut cotyledonary petiole base. This blackening occurred as a result of an interaction between the cells at the cut surface and the culture medium and was termed 'tissue culture blackening'. Cotyledonary petioles, from genotypes that exhibited tissue culture blackening, only blackened when in direct contact with the culture medium (see Figure 4). The degree of blackening was less apparent for hypocotyl explants as these were laid on to the surface of the regeneration medium with the cut surface in contact but not embedded into the medium.

The two parental genotypes showed distinct differences in callus and blackening traits, and within the DH population a small number of genotypes showed responses similar to one or other of the parents. In genotypes that produced callus, the cut surface and callus tended to be green (with slight browning occurring towards the end of the

culture period) whilst genotypes with less callus and swelling, showed more blackening to the base. Genotypes that did not swell at the base, and produced no callus, exhibited tissue culture blackening at the cut surface. These findings suggest that there may be a link between callus formation and tissue culture blackening. However, across the whole population, variation for callus formation and tissue culture blackening could not easily be separated into discrete classes and was therefore difficult to score objectively. The genetic control of these traits was not further investigated within the DH population. The effect of tissue culture blackening, on shoot regeneration potential and ultimately on transformation efficiency is discussed later.

Figure 3 **A comparison of shoot regeneration from cotyledonary petioles and hypocotyl segments of the two parental DH lines.**

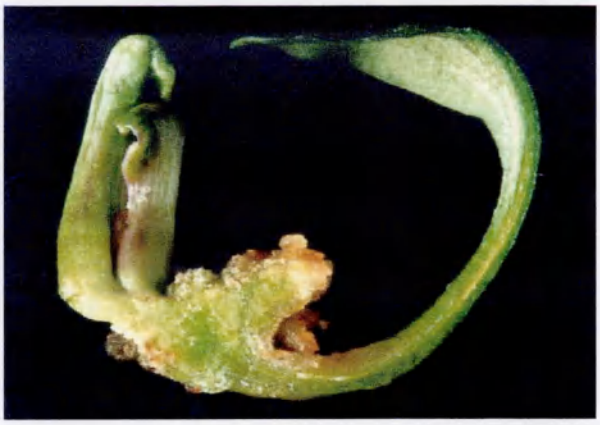
Shoot regeneration from hypocotyl explants of GDDH33 (a) and A12DHd (b), and from cotyledonary petioles (shown in cross-section) of GDDH33 (c) and A12DHd (d). For both hypocotyl and cotyledonary petioles of GDDH33 (a and c), regeneration is via an initial callus phase; whilst shoot regeneration from A12DHd appears to be direct from cells at or near the cut surface (b and d).

Figure 4 **Tissue culture blackening.**

Two cotyledonary petioles from the same genotype (1 and 2): (1) has been embedded in the culture medium and has turned black, while (2) was not embedded and has a 'sugary' callus over the cut surface. This 'sugary' callus is possibly a wound response (to seal the infection site) and indicates that it is a reaction with the medium that causes tissue culture blackening in this genotype.

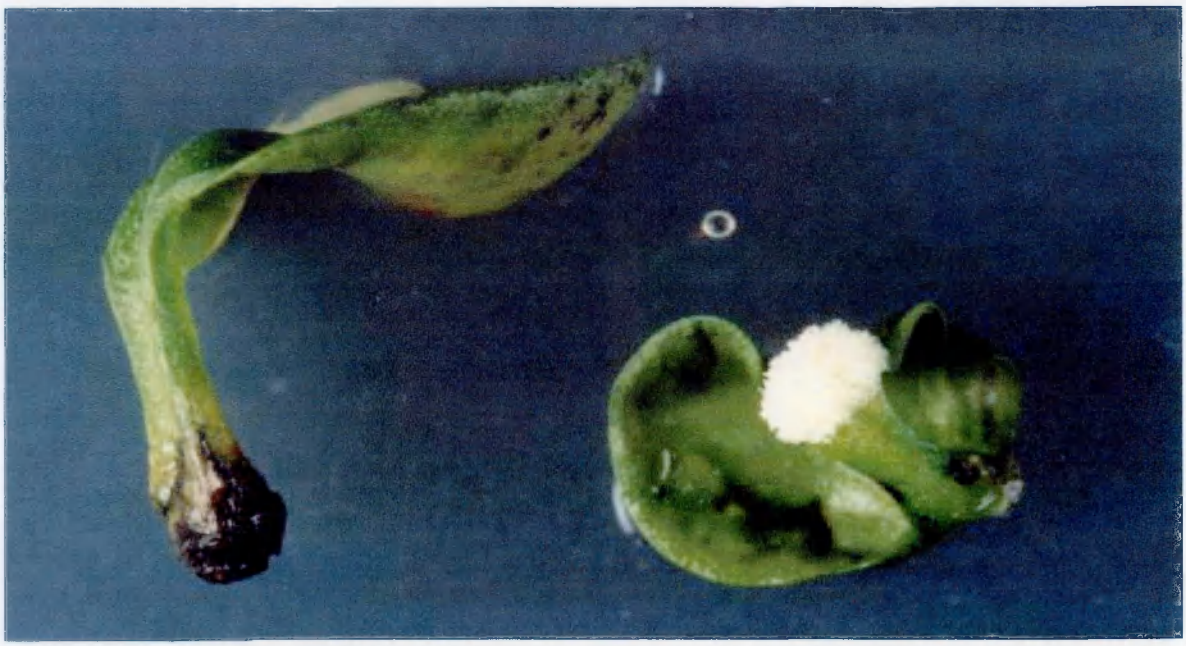
a

b



c

d



1 2

3.2.2.2 Regeneration within the DH mapping population

Both cotyledonary petioles and hypocotyl segments were scored for the presence or absence of regenerating shoots and roots. Only a small number of genotypes produced roots from hypocotyl segments, and of those genotypes that did, regeneration frequencies were not consistent across the replicates (data not shown). Therefore, the variation in root production from hypocotyl explants was not further investigated. The data collected, for shoot and root regeneration from cotyledonary petioles and shoot regeneration from hypocotyl explants, after 44 days in culture, are presented in Table 6.

Table 6 *In vitro* regeneration from cotyledonary petioles and hypocotyl explants after 44 days in culture.

Data are expressed as counts (number of explants regenerating / total number of explants in culture).

DH Line	Shoot regeneration from hypocotyl explants	Shoot regeneration from cotyledonary petioles	Root regeneration from cotyledonary petioles
GDDH33	0.35	0.15	0.03
A12DHd	0.74	0.68	0.04
1002	0.30	0.00	0.06
1004	0.38	0.03	0.03
1011	0.26	0.05	0.25
1012	0.46	0.92	0.54
1017	0.64	0.30	0.59
1020	0.20	0.33	0.13
1021	0.24	0.26	0.31
1027	0.02	0.03	0.03
1035	0.36	0.14	0.00
1036	0.10	0.01	0.11
1039	0.08	0.00	0.09
1042	0.12	0.04	0.31
1058	0.14	0.00	0.02
2056	0.20	0.08	0.21
2068	0.84	0.85	0.10
2069	0.03	0.01	0.03

DH Line	Shoot regeneration from hypocotyl explants	Shoot regeneration from cotyledonary petioles	Root regeneration from cotyledonary petioles
2072	0.06	0.05	0.13
2073	0.18	0.14	0.38
2075	0.26	0.27	0.36
2134	0.22	0.03	0.02
2186	0.49	0.41	0.00
2190	0.04	0.18	0.14
2208	0.72	0.72	0.09
2270	0.24	0.14	0.18
2221	0.08	0.00	0.09
3066	0.22	0.10	0.13
3070	0.55	0.73	0.00
3078	0.48	0.03	0.36
3079	0.08	0.00	0.14
3083	0.06	0.02	0.15
3088	0.28	0.03	0.02
3130	0.06	0.00	0.55
3235	0.12	0.30	0.30
4030	0.52	0.15	0.63
4031	0.14	0.02	0.17
4034	0.26	0.16	0.37
4052	0.54	0.41	0.26
4054	0.14	0.15	0.29
4137	0.04	0.00	0.20
4199	0.54	0.10	0.37
4201	0.18	0.18	0.45
5005	0.38	0.13	0.03
5047	0.56	0.74	0.01
5070	0.45	0.74	0.00
5075	0.50	0.06	0.02
5076	0.02	0.16	0.10
5079	0.24	0.03	0.14
5080	0.28	0.08	0.26
5117	0.68	0.37	0.50
5118	0.06	0.01	0.50
5145	0.20	0.23	0.55
5147	0.02	0.01	0.19
6015	0.46	0.01	0.42
6024	0.44	0.12	0.40
6036	0.02	0.01	0.02

Significant differences were observed between genotypes ($P < 0.001$) for shoot regeneration from both cotyledonary petioles and hypocotyl explants, and for root regeneration from cotyledonary petioles, across the DH population. This confirmed regeneration to be genotype dependent. A correlation coefficient of $r = 0.01$ ($P > 0.05$) was obtained when shoot and root regeneration from cotyledonary petioles were

compared. This highlighted that there was no direct relationship between the two regeneration responses, and that root production was independent of shoot regeneration. It was therefore concluded that root regeneration was not antagonistic to the production of shoots, *in vitro*.

Regeneration rates above and below those of the two parents were observed within the DH population (Figure 5). Both parents regenerated shoots, and within the DH population some lines responded better and some worse than the two parents (*i.e.* the parents did not represent the two extremes of the distribution), as such it could be concluded that a single gene did not control shoot regeneration. Shoot regeneration from the two tissue types fell into 'classes' of non-, low, intermediate and high shoot regenerating genotypes (Figures 5a and 5b). The average shoot regeneration response from hypocotyl explants was 0.28 while regeneration from cotyledonary petioles had an average response of 0.18. One-way ANOVA was carried out on each of the DH lines to further compare the shoot regeneration response from the two tissue types. The data suggested shoot regeneration was under different but possibly linked genetic control, as some DH lines showed no significant difference in regeneration response between the two tissue types, whilst others did. Generally shoot regeneration rates from hypocotyl explants were similar to or higher than rates from cotyledonary petioles (from the same genotype). However, some DH lines that regenerated shoots from hypocotyl explants failed to do so from cotyledonary petioles (Table 6), showing the ability to regenerate from one tissue type did not guarantee regeneration from the other tissue type. A correlation coefficient of $r = 0.66$ ($P < 0.01$) was calculated when shoot regeneration from the two tissue types was compared. Although not directly related (value less than 1) there was still a significant association between shoot regeneration from the two tissue types.

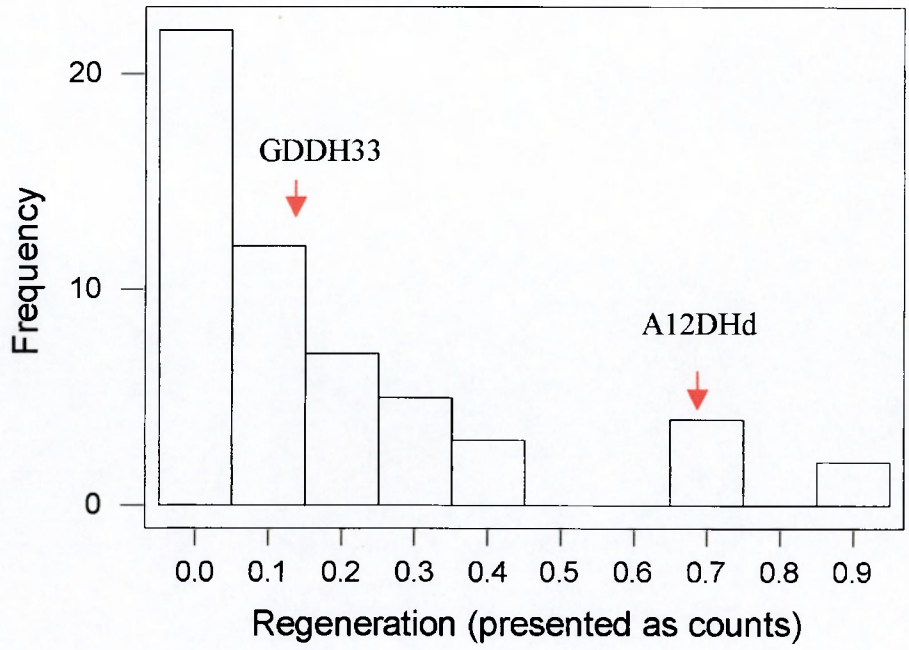
Figure 5 (a) Distribution frequency of shoot regeneration from cotyledonary petioles across the DH population. The data are presented as counts (*i.e.* the number of explants regenerating shoots after 44 days in culture / the total number of explants).

Figure 5 (b) Distribution frequency of shoot regeneration from hypocotyl explants across the DH population. The data are presented as counts (*i.e.* the number of explants regenerating shoots after 44 days in culture / the total number of explants).

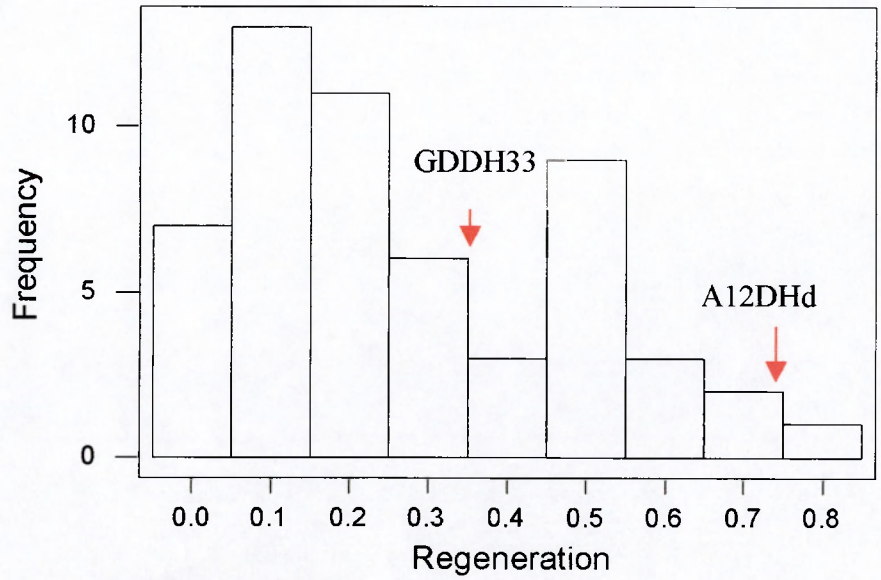
Figure 5 (c) Distribution frequency of root regeneration from cotyledonary petioles across the DH population. The data are presented as counts (*i.e.* the number of explants regenerating roots after 44 days in culture / the total number of explants).

The relative positions of the two parents (A12DHd and GDDH33) are indicated in red.

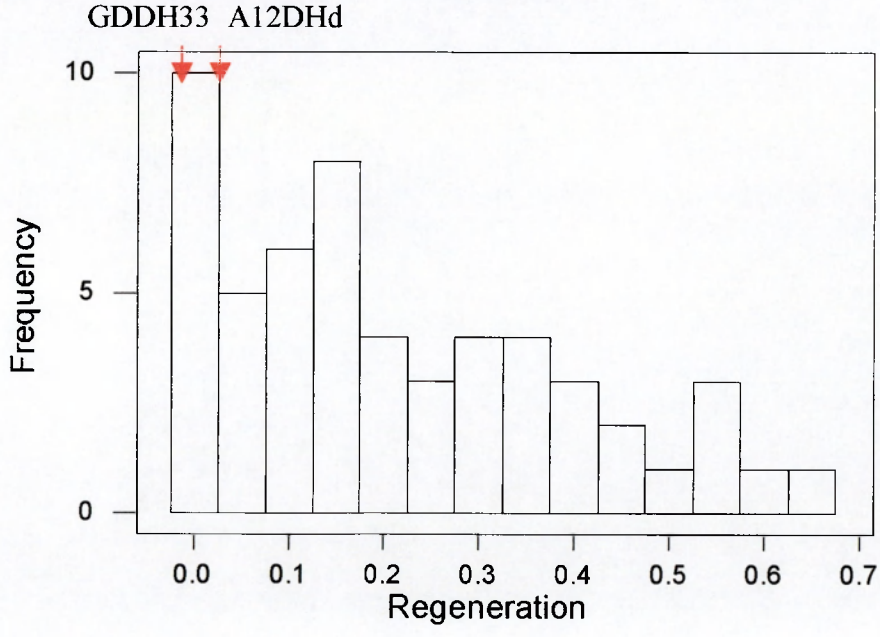
(a)


Frequency
20
10
0
GDDH33
A12DHd
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Regeneration (presented as counts)

(b)


Frequency
10
5
0
GDDH33
A12DHd
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
Regeneration

(c)


GDDH33 A12DHd
Frequency
10
5
0
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7
Regeneration

The totipotency theory suggests that most cells contain the genetic makeup needed to regenerate a whole plant, so why should shoot regeneration be different for the two tissue types? (1) The endogenous hormone levels of the two tissue types may be different at the time of isolation. (2) It may be related to the ability of a cell to dedifferentiate. (3) Under these experimental conditions, cotyledonary explants are more affected by tissue culture blackening. In a number of instances genotypes that regenerated shoots only from hypocotyl explants did exhibit tissue culture blackening from cotyledonary petioles. This suggests that blackening may have an inhibitory effect on shoot regeneration. However, an equally high number of genotypes that regenerated shoots from cotyledonary petioles, at a relatively high frequency, also showed a high level of blackening at the petiole base. So blackening may inhibit or reduce shoot regeneration potential, but will not prevent shooting. As cotyledonary petioles were more prone to tissue culture blackening (due to explants being embedded into the media), this may explain why the overall shoot regeneration response was lower from cotyledonary petioles than hypocotyl explants. However, the average shoot regeneration response of this DH population from cotyledonary petioles (0.18) was in contrast to the distribution frequencies presented in Appendix A (Figure A.2.2) for shoot regeneration from cotyledonary petioles (with an average regeneration response of 0.72)[1]. In the random population of *B. oleracea*, described in Appendix A, distributions were more continuous with a larger proportion of genotypes having a high regeneration potential. Tissue culture blackening was not as prolific in this mixed population of *B. oleracea*, and again suggests that blackening may inhibit shoot regeneration potential.

The distribution frequencies for both shoot and root regeneration from 4 separate trials are given in Appendix B. These trials varied in the size of the population screened, and one trial was subject to uncontrolled environmental changes during the

[1] These experiments were carried out at separate times, and therefore the direct comparison of data should be read with some degree of caution.

trial period (when the culture room thermostat failed over a weekend period, with temperatures rising as high as 30^0C). The data show clearly that screening this population at different times and under different environmental conditions did not alter the distribution of regeneration frequencies. Although regeneration rates went up and down between trials, the relative ranking of individual genotypes within the population remained the same with the highest regenerating lines always remaining high followed by the intermediate and then low regenerating lines. Some genotypes that exhibited very low regeneration responses were sometimes scored as non- regenerating lines in other screenings. By increasing the number of explants screened per genotype this artefact could be removed in subsequent trials. The data shows that although regeneration is under some environmental influence, it is ultimately genetically controlled, with regeneration potentials being conserved across genotypes.

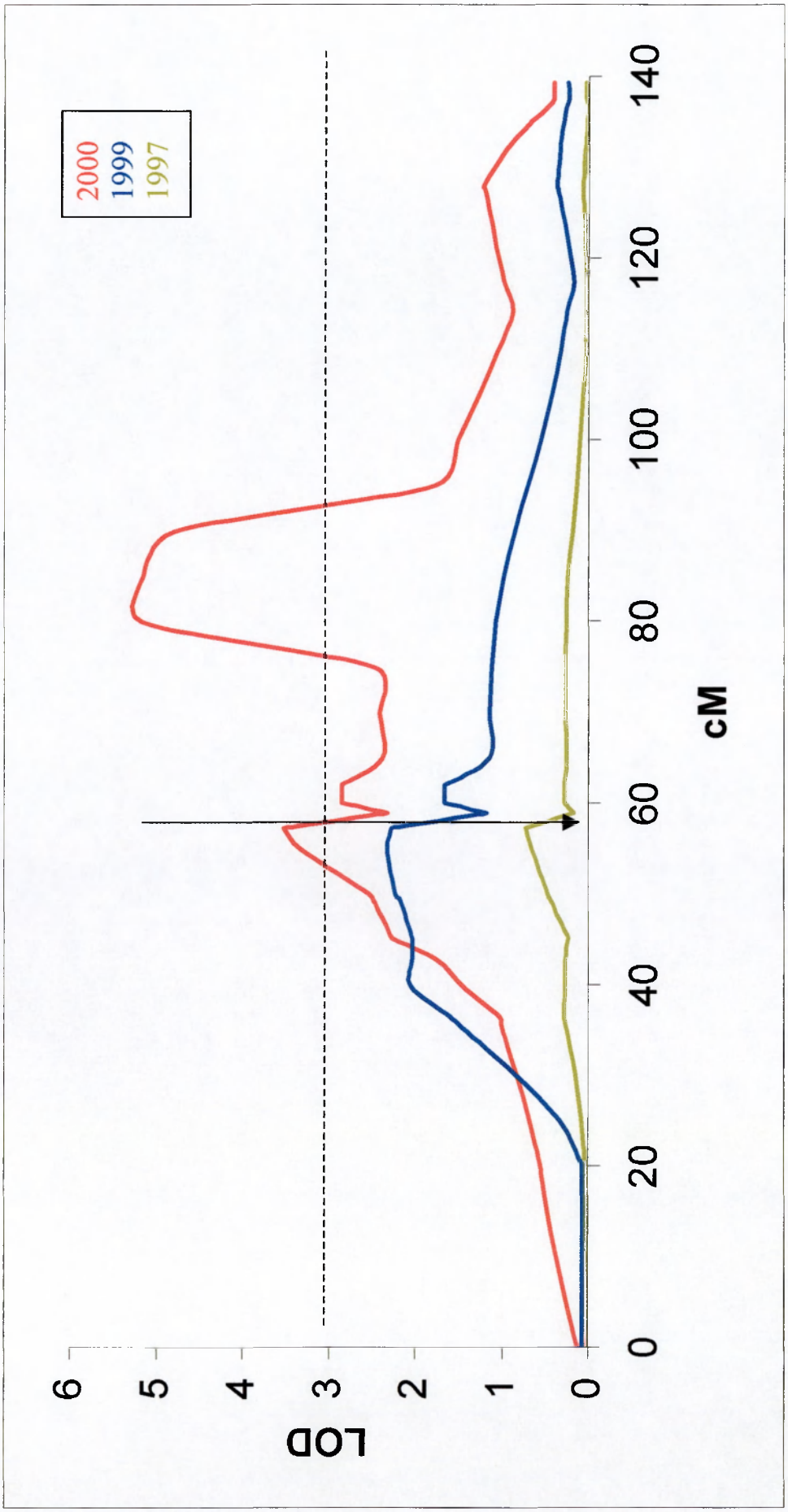
3.2.2.3 Identification of Quantitative Trait Loci (QTL) associated with *in vitro* regeneration

In order to identify the linkage groups and genetic loci associated with the control of shoot and root regeneration the data presented in Table 6 were entered into the QTL mapping programme MapQTL®. The regeneration responses of the DH lines were then compared against the genetic map for this population. Putative QTLs *i.e.* potential regions on the genetic map associated with shoot regeneration from cotyledon and hypocotyl explants, were located on linkage groups 01 and 08 respectively, and for root regeneration from cotyledonary petioles on linkage group 05. The QTL profiles for shoot regeneration from hypocotyl explants and root regeneration from cotyledons both fell below the considered level of significance (Van Ooijen, 1999) with Log-likelihood (LOD) scores of less than 3.0 obtained (data not shown). The QTL profile for shoot regeneration from cotyledonary petioles did rise above the level of significance and is

shown in Figure 6. The QTL profile associated with data from the year 2000 screen showed two peaks above the level of significance. However, the second peak, with a LOD score of 5.2, was not associated with any markers on the genetic map, and was an artefact of the program due to a large barren area with no markers between 64.9 cM and 114.2 cM (refer also to Table 7). Data generated from the 1997 and 1999 screens (discussed in Appendix B) were also entered into MAPQTL®. Although the profiles for each of these years (on linkage group 01) fell below the level of significance, the activity of the profile was similar for all years with a peak found at the same location, 57.2 cM (see Figure 6). The screens over 1997, 1999 and 2000 represented 30, 48 and 55 DH lines respectively. With the increase in population size the significance of this profile has risen. It may be that if more DH lines were screened, this profile (if truly a QTL) may become more significant. Also of note is that in 1999, the trial was subject to unforeseeable environmental changes. This seemed to have had little effect on the overall ranking and variation of the population screened, with high correlation observed when the data collected over the different years was compared.

Figure 6 QTL profiles associated with shoot regeneration from cotyledonary petioles, on linkage group O1.

The QTL profiles associated with shoot regeneration from cotyledonary petioles, over three years (1997, 1999, 2000).


2000
1999
1997
LOD
0
1
2
3
4
5
6
0
20
40
60
80
100
120
140
cM

Single marker analysis was performed using two computer programmes RFLPQB and RFLPRandom (Dr. R. Mithen and Mr. P. Fretter, pers. comm.). This analysis (section 2.9) tests how significantly linked each marker of the genetic map is to the trait under investigation, in this case which of the RFLP markers were most significantly linked to shoot or root regeneration. No markers were significantly associated with shoot regeneration from hypocotyl explants or root regeneration from cotyledonary petioles, and this supports the findings from MAPQTL®. For the putative QTL associated with shoot regeneration from cotyledonary petioles (on linkage group 01) single marker analysis also showed markers on linkage group 01 to be significant, see Table 7, and this supports the findings from MAPQTL®.

These results suggest that genes located on linkage group 01 may be involved in shoot regeneration from cotyledonary petioles. To support and validate these findings with a degree of conviction that would lead to the development of markers suitable for use by breeders, a larger DH population screen and perhaps also an investigation into the elimination of tissue culture blackening would be needed. The screening of a much larger population is not feasible in this particular population, as a number of the original 169 DH lines, used to produce the genetic map, were infertile or highly self-incompatible and have therefore been lost.

It is possible that blackening could be having a suppressing influence on shoot regeneration, to differing degrees, across the whole population. If tissue culture blackening could be removed, shoot regeneration potential might be raised, and if this was the case the QTL profile may again increase in significance.

Table 7 **Single Marker Analysis; to test for significance of markers associated with shoot regeneration from cotyledonary petioles.**

The most significant markers are highlighted in red. The area covered by the putative QTL (see also Figure 6) is highlighted in blue.

Linkage group	cM	LOD	Marker name	t- value †
01	0	0.13	AC-CTCE02	1.18
01	20.1	055	pN186E1N	1.40
01	36.4	1.03	pW239E2	2.30
01	39.8	1.45	p0173E1	3.24
01	42.7	1.76	p043E3	3.11
01	45	2.27	pN52E3NP	2.78
01	45	2.27	p052E3	3.04
01	49.2	2.48	pN107J2	3.16
01	49.9	2.53	pW105E1N	1.23
01	57.2	3.53	pCeriE1	3.09
01	57.2	3.53	pN129E1N	3.22
01	58.9	2.32	AC-CATE13	4.50
01	58.9	2.32	pR36E3	2.40
01	60	2.86	AC-CAAE15	4.50
01	60	2.86	AC-CACE15	4.50
01	60	2.86	pN97J1	2.84
01	60	2.86	p0118E1	1.88
01	61.9	2.86	pN53E2	2.75
01	61.9	2.86	p070J1	2.78
01	64.9	2.38	p0168E1	1.95
01	79.9	5.17	*	*
01	84.9	5.16	*	*
01	114.2	0.88	AC-CACE08	0.68
01	117.4	0.98	AA-CATE17	0.68
01	128.1	1.21	pW216J1	1.21
01	136.6	0.49	pN13E1	1.13
01	137.6	0.41	pR85E1	1.13
01	139.3	0.41	p070E1	1.40

LOD Log likelihood of a particular marker being associated with the trait.

cM Position of marker along the linkage group, in centimorgan.

† Student's t value of ≥ 2.53 were calculated to be significant, at the 0.1% level.

* No associated marker

3.3 12x12 Diallel analysis of shoot and root regeneration

3.3.1 Introduction

The variation observed for both shoot and root regeneration across the DH population verified that regeneration potential is highly genotype dependent. In order to further investigate the control and inheritance patterns for these traits, diallel analysis was carried out. Using the results from the screening of the DH population, 12 DH lines differing in shoot regeneration response were selected for diallel analysis. Lines were primarily selected for shoot regeneration response from cotyledonary petioles, but as far as possible lines were also selected that showed variation in shoot regeneration from hypocotyl explants, and root regeneration from cotyledons.

3.3.2 Method

Both reciprocal crosses and selfed progeny were produced in a 12 x 12 diallel, as described in section 2.1.2. From a possible 132 F_1 combinations, seed was produced in large enough numbers to enable screening of 128 F_1 hybrids for shoot and root regeneration. The 12 DH parental lines (selfed population) were also screened for shoot and root regeneration from both hypocotyl and cotyledonary explants as previously described.

One hundred cotyledonary petioles and 50 hypocotyl explants were established for each of the 140 genotypes screened (128 F_1 and 12 DH lines). Due to the size of the trial the experiment was established over 5 weeks (with 20 cotyledon and 10 hypocotyl explants established each week for each of the genotypes). Cultures were positioned in the culture room (conditions as previously described) using a randomised block design, in order to monitor any potential culture room position effects. Explants were scored

individually after 44 days in culture for (1) the presence or absence of roots and shoots and (2) the number of shoots produced per explant.

Two-way ANOVA, using a random model, was carried out on the data sets to determine the amount of variation ascribed to both genetic and environmental effects. The diallel tables were further analysed using methods described by Hayman (1954) and genetic component analysis was carried out using the methods described by Mather and Jinks (1987). These analyses provide detailed information on the extent and nature of the genetic control of the traits under investigation, and are further described in the relevant sections following.

3.3.3 Results

One hundred and twenty eight F_1 hybrids and 12 DH parents, from the 12 x 12 diallel, were screened for both root and shoot regeneration from cotyledonary petioles and shoot regeneration from hypocotyl segments. Two-way analysis of variance was carried out on each of the data sets to determine the proportion of variation ascribed to both genetic and environmental effects. The proportion of variation associated with non-genetic effects, *i.e.* environmental effects and experimental errors, was less than or equal to 30 % for all traits except shoot regeneration from hypocotyl segments. For this trait 66.3 % of the variation was attributed to environmental and experimental error effects and suggests that the genetic control for this trait is weak. It also suggests that the experimental conditions for shoot regeneration from hypocotyl explants were not favourable and / or hypocotyl explants were more sensitive to these environmental conditions. The results from further biometrical analysis, which was carried out on the hypocotyl data set, were therefore interpreted with caution.

3.3.3.1 Shoot regeneration from cotyledonary explants

The 12 DH parental lines and 128 F_1 hybrids were scored for the presence or absence of shoots after 44 days in culture. Shoot regeneration rates (expressed as mean counts) are presented in Table 8. The ability to regenerate shoots was seen to be a heritable trait. This was clearly shown when a non- (or low) regenerating line was crossed with a higher regenerating line; the regeneration response in the resulting F_1 hybrid was significantly higher than that of the lower regenerating parent, see also Figure 7. This demonstrates the potential to introduce regeneration ability into recalcitrant lines, by sexual hybridisation, and suggests that high regenerating genotypes are dominant over low.

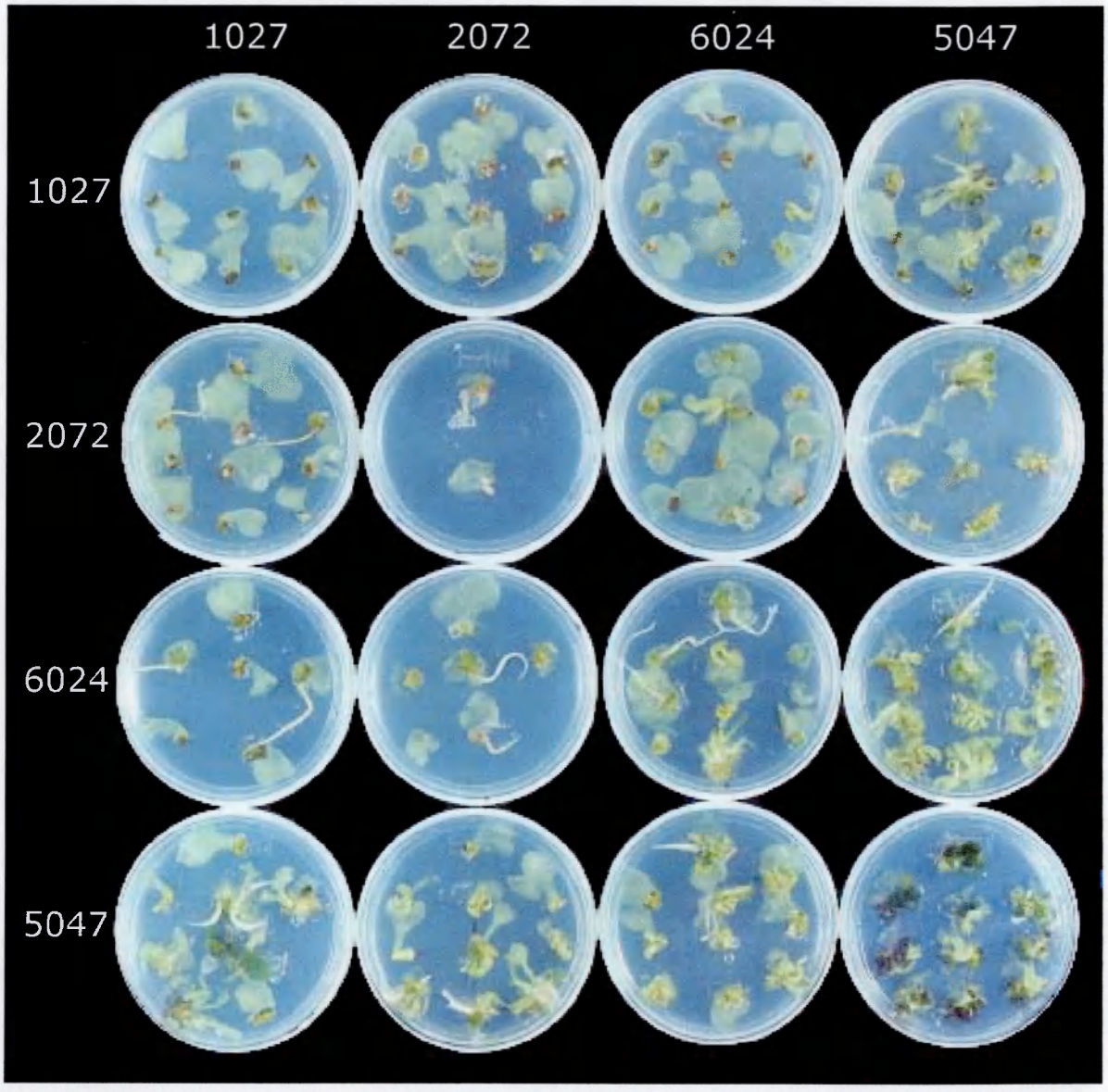
Table 8 Diallel table of shoot regeneration (expressed as mean counts) from cotyledonary petioles, after 44 days in culture

Male / Female	5070	3070	5047	5117	4052	6024	4030	2072	5118	2069	1027	1002
5070	**1.00**	1.00	1.00	1.00	0.98	0.99	0.93	0.99	0.95	0.98	0.68	0.94
3070	1.00	**1.00**	0.98	1.00	1.00	1.00	0.98	1.00	1.00	1.00	0.89	0.86
5047	1.00	0.98	**0.99**	0.99	0.94	0.98	0.98	0.94	0.97	0.77	0.80	0.95
5117	1.00	1.00	0.99	**0.84**	0.90	0.71	0.92	0.71	0.55	0.77	0.53	0.49
4052	0.98	1.00	0.98	0.90	**0.90**	0.80	0.96	0.70	0.53	0.88	0.62	0.74
6024	0.99	1.00	1.00	0.88	0.80	**0.40**	0.67	0.66	0.61	0.73	0.09	0.57
4030	0.94	0.99	0.92	0.92	0.89	0.34	**0.30**	0.61	0.56	0.32	0.22	0.41
2072	0.99	1.00	0.94	0.89	0.56	0.53	0.62	**0.31**	0.20	0.46	0.04	0.15
5118	0.95	1.00	0.97	0.55	0.53	0.61	0.56	0.20	**0.18**	0.70	0.13	0.08
2069	0.97	1.00	0.61	0.71	0.81	0.73	0.38	0.42	0.68	**0.10**	0.08	0.12
1027	0.79	0.89	0.92	0.72	0.70	0.27	0.24	0.04	0.13	0.06	**0.01**	0.00
1002	0.94	0.86	0.95	0.49	0.86	0.58	0.30	0.15	0.08	0.12	0.00	**0.02**

N.B. where no F_1 seed was available, as indicated in blue, the results from the reciprocal cross was used. The regeneration responses of the parental lines are shown in red.

Figure 7 **Photograph showing inheritance of shoot regeneration in a 4 x 4 subset of the diallel.**

Plates have been photographed from the underside to better illustrate shoot regeneration from the petiole base. The four DH parental lines, 1027, 2072, 6024 and 5047, all differed in regeneration potential (0.01, 0.31, 0.40 and 0.99 respectively). The inheritance of shoot regeneration is best shown in the DH 1027 array; here shoot regeneration is shown to increase in the F_1 represented across and down in this array, with shoot density increasing when DH 1027 is crossed to higher and higher regenerating lines.


1027
2072
6024
5047
1027
2072
6024
5047

Two-way ANOVA was carried out on data from the diallel to estimate the degree of genetic control of this trait. Analysis suggested that just 15 % of the variation observed within the diallel table was a result of non-genetic or environmental effects, and that 85 % of the variation was due to genetic effects. Data from the diallel cross were analysed further using methods described by Hayman (1954) and Mather and Jinks (1987). These analyses provide information about the average additive effects of the genes, about the effects of heterozygosity, about the dominance of the genes in each parent, and about gene interaction. High expression of a character in a cross can be attributed either to heterozygosity (which will not be fixed in the population, due to subsequent segregation); to additive gene action, *i.e.* to the combined effect of a number of genes all acting positively to increase expression of the character; or to non-additive gene action, *i.e.* to an interaction between genes (epistasis) if the interaction is between separate loci or to dominance relationships if non-additivity is within loci. It can also be decided, in those cases where dominance relationships are effective, whether the high expression is due to dominant genes, to recessive genes or to a balanced grouping of dominant and recessive genes. Furthermore the analysis should demonstrate whether any of the DH parents contain the complete complement of dominant or recessive genes.

Analysis of variance of the diallel table, following Hayman (1954), revealed that both additive (a) and dominant (b) gene effects were significant for shoot regeneration from cotyledonary petioles, at the 0.1 % level (Table 9), with additive gene effects being more important.

Table 9 **Analysis of variance of the 12 x 12 diallel, table for shoot regeneration from cotyledonary petioles, after Hayman (1954), calculated using Genstat version 5.0.**

Item	MS	df	F	
a	5.1299	11	288	***
b_1	2.2933	1	129	***
b_2	0.0532	11	2.99	**
b_3	0.2547	54	13.8	***
b	0.2520	66	14.16	***
c	0.0225	11	1.26	n.s
d	0.0128	55	0.72	n.s
block error	0.0178	572		

Significance level
** 1.0 %
*** 0.1 %
n.s = not significant

(Where (a) is additive, (b) is dominance and (c and d) are maternal effects)

The relatively high b_1 mean square (MS) indicates directional dominance and in comparing the mean of the F_1 crosses (0.71) and the mean of the DH selfs (0.50), from the diallel, it can be seen that there is dominance for high shoot regeneration. Furthermore, the significance of b_2 indicates that the extent of directional dominance varies between the 12 DH parents reflecting the fact that they carry different numbers of dominant alleles. b_3 is an estimate of the residual dominance (*i.e.* the dominance that is not accounted for by directional dominance b_1 and ambidirectional dominance b_2). Although all forms of b proved statistically significant, the high MS of directional dominance, b_1, would suggest this to be the most significant of the dominance values and this is supported in subsequent analysis. Both maternal effects (c and d) were not significant, suggesting no significant differences were observed between reciprocal crosses. This justifies the use of reciprocal values (highlighted in blue in Table 8) where missing values were present.

The relationship between the variance of the F_1 offspring to the recurrent parental line (Vr) and their covariance with the non-recurrent parent (Wr) for shoot regeneration is shown in Figure 8. The regression graph of Vr and Wr for arrays in diallel crosses provides a useful means of assessing the genetic relationship among homozygous

parents. To better explain the Vr and Wr terms, consider the array of genotype A in a 4 x 4 diallel.

Male Female	A	B	C	D
A	A x A	A x B	A x C	A x D
B	B x A			
C	C x A			
D	D x A			

To calculate the variance (Vr) of array A, the total variance of each F_1 hybrid in this array (indicated in blue) and parent A is calculated. Variance is the mean of the squared deviation and deviation is the amount the F_1 hybrid mean differs from the parental mean, in this case A. The stronger the effect of A the smaller the variance observed between F_1 offspring and the recurrent parent (A) *i.e.* the F_1 will have a value similar to parent A. Conversely if the variance (Vr) is high, the F_1 are very different from the parental value which suggests the recurrent parent phenotype to be recessive. Wr is the co-variance of a particular array and the non-recurrent parent, in the above example that would be the variance of the F_1 highlighted in blue and the associated other parent (*i.e.* not parent A).

In Figure 8 the relationship between the variance of the F_1 hybrids for each parental line (Vr) and their covariance with the non recurrent parent (Wr) is shown. The graph of Vr and Wr provides information on three points. First it supplies a test for the adequacy of the model (the assumption being that genetic control is due to an additive-dominance model with additive environmental effects and independence of the genes in action and in distribution among the parents). If this model is adequate, then the linear regression of Wr on Vr, has a slope of 1.0. Second, given that the model is adequate, a measure of the degree of dominance is provided by the departure from the origin where

the regression line cuts the Wr axis. Cutting the axis significantly above the origin suggests incomplete dominance of the trait (and additive effects are thus likely to be predominant) and cutting the axis significantly below the origin would suggest over-dominance of the trait. Finally the relative order of the points along the regression line indicates the distribution of dominant and recessive genes among the parents. In Figure 8, the slope of the Wr and Vr regression line was 0.95 and thus, was not significantly different to the line of unity (1.0), allowing the model to be further analysed.

The DH lines that fall close to the origin had the smallest Vr and Wr values (DH 3070, 5070 and 5047); these lines, therefore, have more dominant alleles while those further from the origin have more recessive alleles. The high regenerating lines had the smallest Vr and Wr values and this indicates that the ability to regenerate shoots at a high frequency is dominant over low regeneration ability. Figure 8 shows dominance of the shoot regeneration trait to be unidirectional. The high shoot regenerating lines had the smallest Vr and Wr values; the low regenerating lines had the highest Vr and Wr values and the intermediate regenerating lines had values in between the two phenotypes. This supports the data presented in Table 9 (ANOVA of the diallel table) with b_1 showing high significance.

The limiting parabola is also shown in Figure 8 (calculated by $\sqrt{Vr \times Vp}$ where Vp is the variance of the parents). The closer the line of Vr, Wr to the curve of the limiting parabola the more incomplete the dominance effect. Completely recessive parents correspond to the upper end of the regression line, where it cuts the limiting parabola and completely dominant parents to the lower end of the regression line where it cuts the limiting parabola. DH lines 1002 and 1027 (both low shoot-regenerating lines) are associated with the upper end of the regression line where it cuts the limiting parabola; these lines therefore have more recessive alleles for shoot regeneration. DH lines 5070, 3070 and 5047 (all high regenerators) were associated at the lower end of the regression

line where it cuts the limiting parabola and show dominance for increased shoot regeneration from cotyledonary petioles. The intercept of the regression line was above the origin and indicates incomplete dominance for shoot regeneration, and thus supports the suggestion that additive gene effects play a role in the genetic control of shoot regeneration.

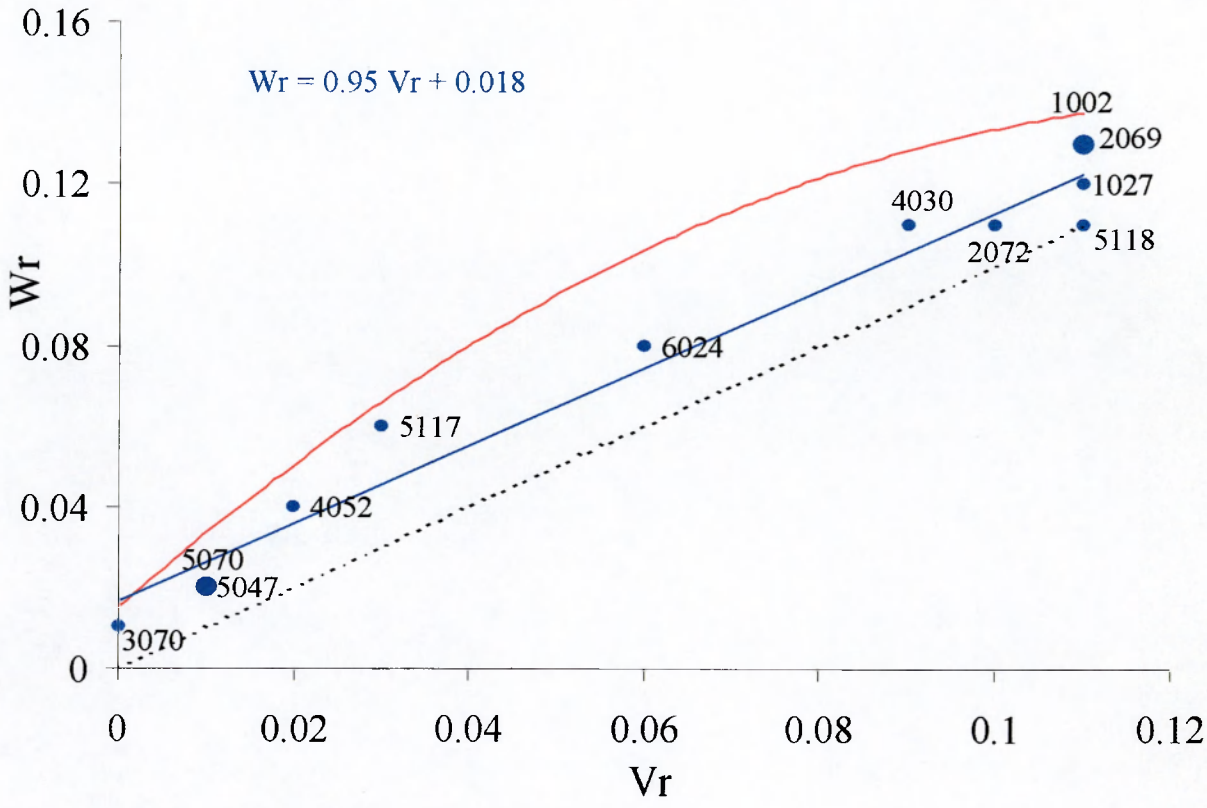
A plot of Wr + Vr against the mean parental value is shown in Figure 9. The higher the shoot regeneration the smaller the corresponding Wr + Vr, again showing that the alleles for high shoot regeneration are dominant to those associated with low shoot regeneration. The plot of Wr + Vr against the mean parental value gave a positive correlation coefficient of $r = 0.97$ ($P < 0.01$) indicating that dominant alleles act to increase expression of the character, in this case high shoot regeneration. The r-value of 0.97 also suggests that dominance is unidirectional. An r-value of 1 is indicative of unidirectional dominance, while an r-value of 0 would suggest ambidirectional dominance *i.e.* a random relationship. All points also lie close to the line, indicating that the dominance relationship holds true for all parents.

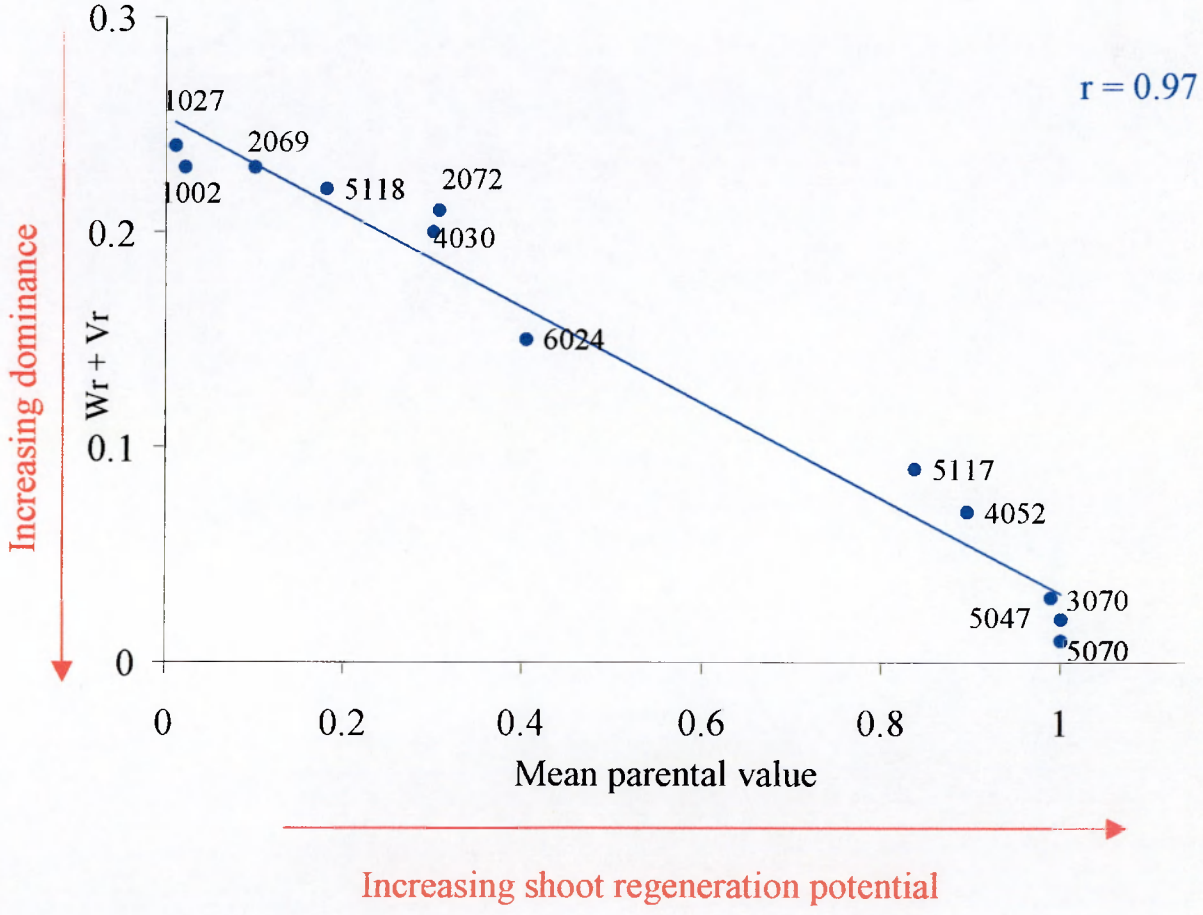
Figure 8 **The relationship between the variance of the F_1, for each parental line (Vr) and their covariance with the recurrent parent (Wr), for shoot regeneration from cotyledonary petioles.**

● Plot of Vr vs Wr. The red line is that of the limiting parabola = $\sqrt{Vr.Vp}$ (where Vp is the variance of the parents). The dashed line indicates the theoretical line of unity.

Figure 9 **Wr + Vr from each array of the 12 x 12 diallel plotted against the mean of the common parent.**

All points lie close to the line, indicating that the dominance relationship (for shoot regeneration from cotyledonary petioles) holds true for all parents.


Wr = 0.95 Vr + 0.018
0.16
0.12
0.08
0.04
0
Wr
0
0.02
0.04
0.06
0.08
0.1
0.12
Vr
1002
2069
1027
4030
2072
5118
6024
5117
4052
5070
5047
3070


r = 0.97
0.3
0.2
0.1
0
Wr + Vr
0
0.2
0.4
0.6
0.8
1
Mean parental value
Increasing dominance
Increasing shoot regeneration potential
1027
2069
1002
5118
2072
4030
6024
5117
4052
3070
5047
5070

The relationship between Vr and Wr for shoot regeneration from cotyledonary petioles gave no reason to doubt the adequacy of the simple model. Therefore, the components of variation (D, H_1, H_2, F and E) were calculated to further investigate the genetic control of shooting from cotyledonary petioles. The genetic components analysis of shoot regeneration from cotyledonary petioles are presented in Table 10 (and the formulas for their calculation are presented in Appendix C).

Table 10 Genetic Components Analysis: Shoot regeneration from cotyledonary petioles.

Component	
D	0.148
H_1	0.056
H_2	0.066
F	- 0.025
E	0.018
Mean degree of dominance $\sqrt{H_1/D}$	0.615
Proportion of dominance $H_2/4H_1$	0.295
Broad-sense heritability	0.849
Narrow-sense heritability	0.709

For the inheritance of shoot regeneration potential, additive genetic variation (D) was larger than the dominance genetic variances (H_1 and H_2). This was also noted in Table 9 with F values far greater for additive effects (a) than dominant effects (b). D values greater than H_1 again indicate incomplete dominance (which is expected if additive effects play a major role). The dominance genetic variance H_2 was approximately equal to H_1, suggesting there to be equal allele frequencies, *i.e.* $u = v$ at the relevant loci. The mean value of uv over all these loci, estimated from the ratio $H_2/4H_1$, was 0.295 which approximates to the maximum value of 0.25 which arises when u = v = 0.5 at all loci. The mean degree of dominance ($\sqrt{H_1/D}$) was 0.615 again indicating incomplete dominance (a value of 1.0 would indicate complete dominance and a value of >1 would indicate over dominance). This confirmed the results of the

graphical analysis (Figure 8) with the intercept being above the origin. The broad and narrow sense heritabilities were 0.849 and 0.709 respectively. Broad sense heritability is based on the ratio of total genetic variation to the total phenotypic variation. Therefore, for shoot regeneration from cotyledonary petioles around 85 % of the phenotypic variation will be heritable with the remaining 15 % being associated with environmental or non-heritable effects. Narrow sense heritability is more important as it provides a measure of the breeding value of a population, and measures the proportion of the variation that is due to the additive gene effects of genes in the population. The high narrow sense heritibility value of 0.709 shows that around 71 % of this trait is controlled by additive gene effects, and therefore the potential to introduce this trait into breeding material is obviously high.

In summary, high shoot regeneration was observed to be dominant over low shoot regeneration. Crossing high and low phenotypes together results in a shoot regeneration potential in the F_1 hybrid, at a rate above that of the low regenerating parent, see Figure 7. The genetic control of this trait is a result of both additive and dominant effects, with additive effects being stronger. For breeding purposes a strong additive effect is desirable if this trait is to be introduced into subsequent populations.

3.3.3.2 Inheritance of Multiple Shoot regeneration potential

In order to investigate if the regeneration of multiple shoots was dominant over the regeneration of just one or a few shoots per explant, the number of shoots regenerating from each cotyledon was also scored. Here genotypes were scored for the total number of shoots formed / the number of explants that were shooting. A score of 8 shoots per explant was set as the maximum, as numbers greater than this were hard to score accurately. The average score for the five replicate screens are presented in Table 11.

Table 11 Diallel table showing number of shoots formed per cotyledon / the number of explants shooting, after 44 days in culture.

Male / Female	5070	3070	5047	5117	4052	6024	4030	2072	5118	2069	1027	1002
5070	**6.63**	8.00	8.00	6.31	6.61	6.73	5.10	6.43	5.32	4.99	4.46	5.45
3070	8.00	**7.89**	7.40	7.93	7.04	7.57	6.62	8.00	4.67	7.49	5.10	6.00
5047	8.00	7.40	**7.64**	6.20	6.89	6.36	7.59	6.71	5.31	5.03	4.54	6.07
5117	6.14	7.93	6.20	**4.49**	5.34	3.53	4.54	3.85	4.27	3.78	2.90	3.04
4052	6.69	7.04	6.41	5.34	**4.21**	4.84	6.17	4.43	3.08	4.14	3.99	4.79
6024	6.73	7.57	6.52	3.67	5.02	**3.80**	3.14	3.33	2.79	3.91	1.13	3.33
4030	4.90	6.62	5.68	4.54	5.20	2.85	**3.15**	3.71	3.07	2.65	2.43	3.42
2072	6.43	8.00	5.87	4.62	3.43	3.38	3.90	**3.53**	2.00	3.12	0.88	2.58
5118	5.32	4.67	5.31	4.27	3.08	2.79	3.07	2.00	**2.18**	2.81	1.98	2.30
2069	5.16	7.55	4.05	3.62	4.30	3.91	3.55	2.62	2.42	**1.89**	1.30	1.73
1027	4.46	5.10	6.47	2.99	4.53	2.05	4.00	0.88	1.98	1.60	**0.60**	0.00
1002	5.45	6.00	6.07	3.04	4.06	2.99	2.32	2.58	2.30	1.73	0.00	**0.20**

A score of 8.0 is indicative of a multiple shoot regenerating line. The regeneration responses of the parental lines are shown in red, and where no F_1 seed was available, as indicated in blue, the results from the reciprocal cross have been used.

Multiple shoot regeneration appears to be associated with high shoot regeneration (when scored as just presence or absence of shoots) with the ordering of the DH parental lines similar in both Table 8 and Table 11, and a high correlation coefficient of $r = 0.8$ ($P < 0.01$) was observed between the two tables. Two-way ANOVA was carried

out to estimate the degree of genetic control of shoot regeneration, when scored for the number of shoots regenerating per explant. Analysis suggested that just 22 % of the variation observed within the diallel table, was a result of non-genetic or environmental effects, and that 78 % of the variation was due to genetic effects. Analysis of variance of the diallel table, following Hayman (1954), revealed that both additive (a) and dominant (b) effects were significant for the genetic control of multiple shoot regeneration from cotyledonary petioles (Table 12), with additive effects being more important. The high MS of b_1 (much higher than that of b_3) indicates directional dominance and in comparing the mean of the F_1 crosses (4.6) with the mean of the DH parents (3.9) from the diallel table, it can be seen that there is dominance for multiple shoot regeneration. Maternal effects (c) and (d) were non-significant.

Table 12 ANOVA of the 12 x 12 diallel for multiple shoot regeneration.

Item	MS	df	F
a	226.15	11	195 ***
b_1	30.55	1	26.3 ***
b_2	2.18	11	1.88 n.s
b_3	5.26	54	4.54 ***
b	5.13	66	4.42 ***
c	1.26	11	1.09 n.s
d	0.61	55	0.53 n.s
block	1.16	572	
error			

Significance level

*** 0.1 %
n.s = not significant

The relationship between the variance of the F_1 offspring to the recurrent parental line (Vr) and their covariance with the non-recurrent parent (Wr) for multiple shoot regeneration is shown in Figure 10. The slope of the regression line for the Wr Vr graph was 0.91, which again was not significantly different from unity, and supports the simple model of additive-dominant genetic control. The smaller Wr and Vr values were

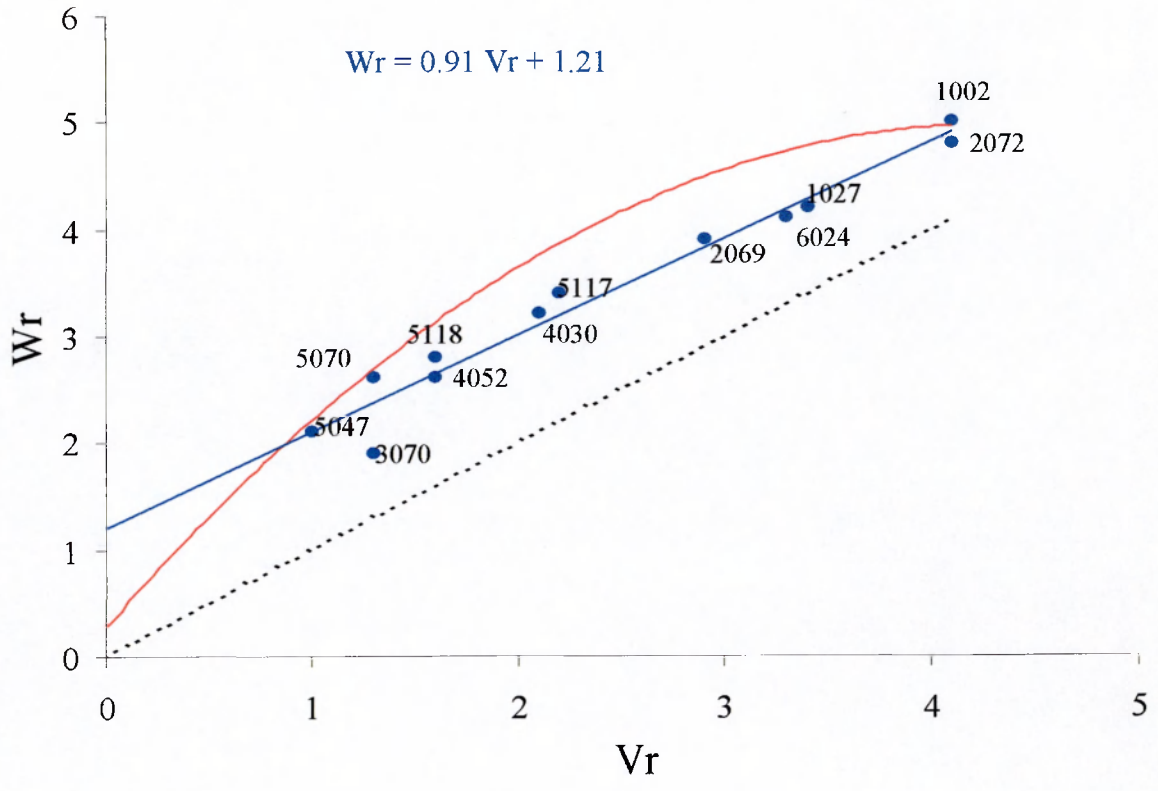
associated with those DH lines that regenerated multiple shoots. These were the same DH lines that showed small Vr and Wr values associated with the dominance of the high shoot regeneration phenotype. The slope of the regression line intercepted the Wr axis at a level significantly above the origin, indicating incomplete dominance of the trait. As the regression line fell close to the line of the limiting parabola this strongly suggests that the majority of the genetic control is due to additive gene effects. The relationship between Wr + Vr and the mean common parent is shown in Figure 11. DH lines that regenerated multiple shoots had the smallest Wr + Vr values, again showing that the alleles for multiple shoot regeneration were dominant to those associated with the regeneration of one or few shoots, however, not all points lay close to the line. This indicated that the dominance relationship does not hold true for all parents (in particular DH 2072) and supports the interpretation of Figure 10, that additive gene effects play the major role. The plot of Wr + Vr against the mean parental value gave a positive correlation coefficient of $r = 0.6$ ($P < 0.01$) indicating that dominant alleles act to increase expression of the character, in this case dominant alleles are associated with multiple shoot regeneration.

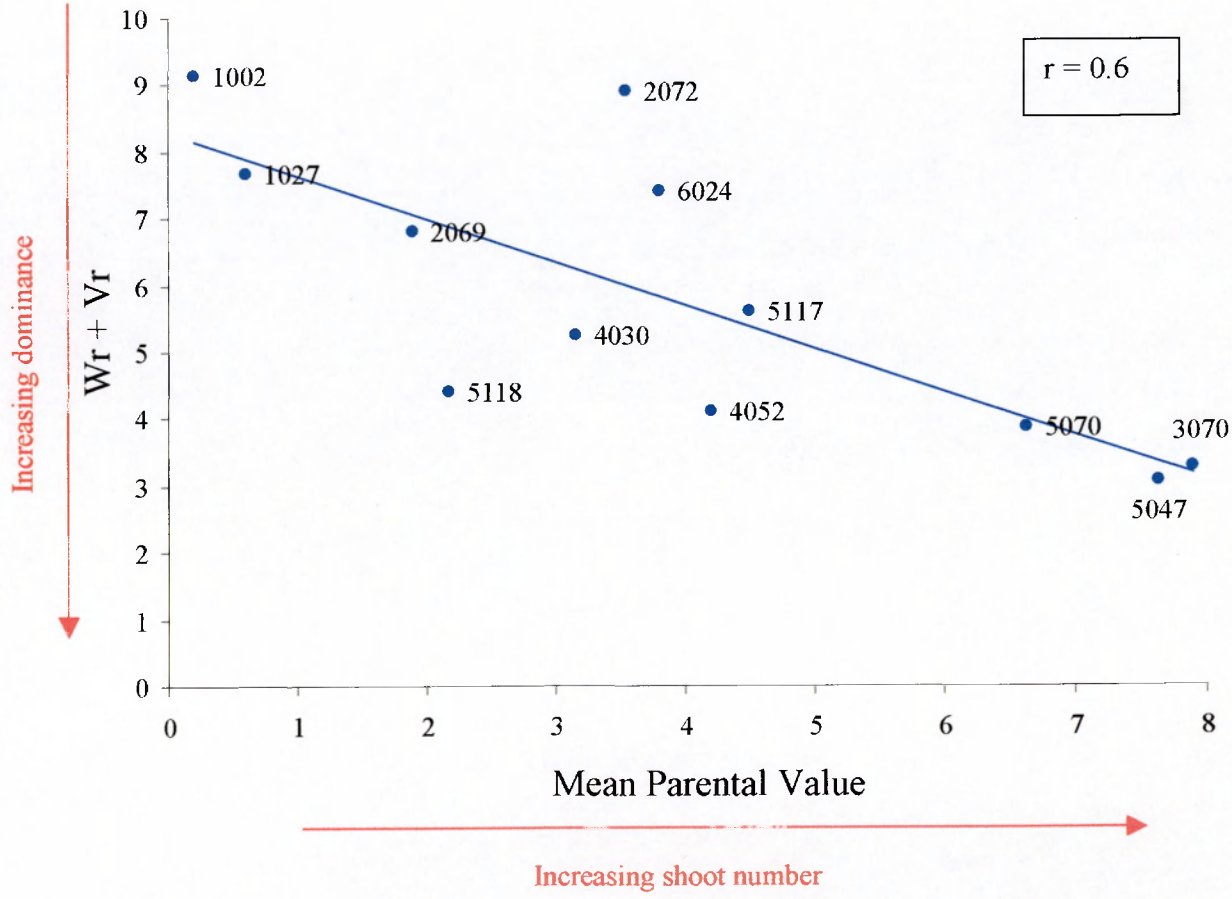
Figure 10 **The relationship between the variance of the F_1, for each parental line (Vr) and their covariance with the recurrent parent (Wr) for multiple shoot regeneration from cotyledonary petioles.**

● Plot of Vr vs Wr. The red line is that of the limiting parabola = $\sqrt{Vr.Vp}$ (where Vp is the variance of the parents). The dashed line indicates the theoretical line of unity.

Figure 11 **Wr + Vr from each array of the 12 x 12 diallel plotted against the mean of the common parent, for multiple shoot regeneration from cotyledonary petioles.**

Not all points lie close to the line, indicating that the dominance relationship for multiple shooting does not holds true for all parents.


Wr = 0.91 Vr + 1.21
1002
2072
1027
6024
2069
5117
4030
5118
5070
4052
5047
3070
Wr
Vr
0
1
2
3
4
5
6


r = 0.6
1002
2072
1027
6024
2069
5117
4030
5118
4052
5070
3070
5047
Wr + Vr
Mean Parental Value
Increasing dominance
Increasing shoot number
0
1
2
3
4
5
6
7
8
9
10

The genetic components of variation D, H_1, H_2, F and E were calculated to further investigate the genetic control of multiple shooting from cotyledonary petioles, and are presented in Table 13. This analysis supported the theory that multiple shoot regeneration was controlled almost entirely by additive gene effects.

Table 13 Genetic Components Analysis: Multiple shoot regeneration from cotyledonary petioles.

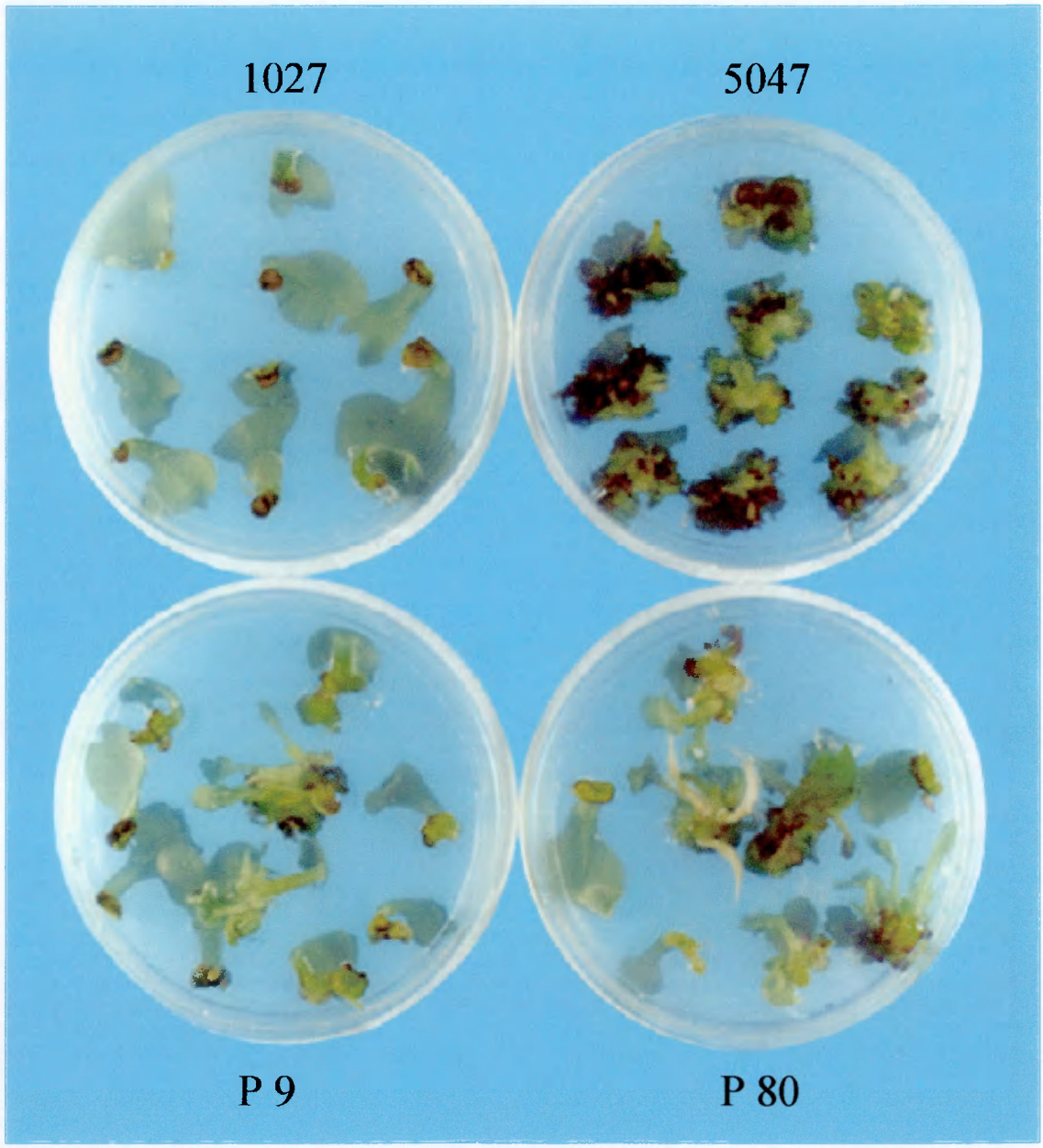
Component	
D	5.22
H_1	0
H_2	0
F	-2.64
E	1.15
Mean degree of dominance $\sqrt{H_1/D}$	-
Proportion of dominance $H_2/4H_1$	-
Broad-sense heritability	0.77
Narrow-sense heritability	0.77

The methods used to calculate the genetic components of variation for multiple shoot regeneration (detailed in Appendix C), show additive gene effects (D) to be highly significant. Under this analysis dominance effects became non significant (H_1 and H_2). The high narrow sense heritability value accounts for all of the broad sense heritability, and suggests that 77 % of the variation observed within the diallel for multiple shoot regeneration was controlled by additive gene effects, with 23 % of the variation accounted for by non-genetic or environmental effects. The high level of additive gene effects controlling this trait means the potential for introducing this trait into desirable material is high. In Figure 12 the inheritance of high multiple shoot regeneration is demonstrated. In this figure, DH 1027 a low regenerating line that produces few shoots per explant (mean value of 0.20) was crossed with DH 5047 a high regenerating line that produces multiple shoot (mean value of 7.64). High shoot regeneration was shown

to be passed on to both the F_1 hybrids, and multiple shooting potential was also shown to be inherited (mean values of 6.47 and 4.54 in the two reciprocal F_1 crosses).

Figure 12 Inheritance of high, multiple shoot regeneration potential.

Figure 12 shows cotyledonary petioles maintained on regeneration medium (with the plates photographed from underneath to better illustrate shoot regeneration). DH 1027 a low regenerating line (mean regeneration frequency 0.01) produced few shoots per explant, with a mean multiple shooting frequency of 0.20, was crossed with DH 5047 a high, multiple shoot, regenerating line (mean regeneration frequency 0.99) with a multiple shooting frequency of 7.64. The resulting F_1 hybrids (P9 and P80) both regenerate shoots (at 0.92 and 0.80 respectively) at a level approaching that of the high regenerating parent. Multiple shooting was observed in a number of F_1 explants that regenerated shoots, at frequencies of 6.47 and 4.54 for P9 and P80 respectively.


1027
5047
P 9
P 80

3.3.3.3 Shoot regeneration from hypocotyl explants

The 12 DH parental lines and 128 F_1 hybrid lines were scored for the presence or absence of shoots regenerating from hypocotyl explants after 44 days in culture. Shoot regeneration rates (expressed as mean counts) for the 128 F_1 and 12 DH lines are presented in Table 14. The parental array in this table has been kept in the same order as in Table 8 (the diallel table for shoot regeneration from cotyledonary petioles). It can be seen that lines DH 1027, 5118, 2069 and 1002, are again low regenerating lines (as Table 8), but the lines that were the high and intermediate regenerating lines, from cotyledonary petioles, have changed order in Table 14. The correlation coefficient between shoot regeneration from cotyledonary petioles and hypocotyl segments was 0.60 ($P < 0.01$), which suggests some association, but also a possible difference in inheritance patterns, and supports the findings from the DH population screen.

Table 14 Diallel table of shoot regeneration (expressed as mean counts) from hypocotyl explants, after 44 days in culture.

Male / Female	5070	3070	5047	5117	4052	6024	4030	2072	5118	2069	1027	1002
5070	**0.30**	0.54	0.88	0.58	0.71	0.50	0.46	0.27	0.10	0.24	0.04	0.40
3070	0.44	**0.59**	0.45	0.92	0.46	0.45	0.40	0.50	0.00	0.46	0.74	0.60
5047	0.33	0.45	**0.54**	0.53	0.38	0.64	0.19	0.36	0.22	0.54	0.17	0.44
5117	0.56	0.79	0.53	**0.68**	0.70	0.44	0.62	0.62	0.31	0.33	0.40	0.63
4052	0.67	0.46	0.55	0.70	**0.39**	0.50	0.63	0.20	0.24	0.12	0.14	0.40
6024	0.50	0.45	0.44	0.51	0.28	**0.51**	0.44	0.20	0.25	0.14	0.19	0.44
4030	0.61	0.40	0.38	0.62	0.72	0.71	**0.38**	0.44	0.29	0.13	0.04	0.33
2072	0.27	0.60	0.32	0.47	0.32	0.16	0.53	**0.20**	0.14	0.12	0.31	0.33
5118	0.10	0.00	0.22	0.32	0.24	0.28	0.29	0.14	**0.10**	0.31	0.25	0.18
2069	0.31	0.82	0.33	0.30	0.36	0.17	0.36	0.04	0.55	**0.07**	0.04	0.12
1027	0.04	0.74	0.50	0.58	0.35	0.29	0.28	0.31	0.25	0.16	**0.04**	0.21
1002	0.40	0.60	0.44	0.63	0.28	0.32	0.33	0.33	0.18	0.12	0.21	**0.15**

Where no F_1 seed was available for analysis, the reciprocal F_1 value was used instead (indicated by numbers in blue). The parental values are shown in red.

The ability to regenerate shoots from hypocotyl explants is again shown to be heritable (Figure 15). Crossing a genotype with low shoot regeneration potential with a genotype with high regeneration potential results in a regeneration response in the F_1 hybrid, often at a rate higher than that of the lower regenerating parent. In some cases the regeneration response in the F_1 hybrid can be at a rate approaching, or higher, than that of the higher regenerating parent. This may be an indication of heterosis, or hybrid vigour, and is associated with increased heterozygosity (the condition of having one or more pairs of dissimilar alleles).

Two-way ANOVA on this data set was discussed in section 3.3.3. This analysis indicated that 66.3 % of the variation observed between genotypes in the 12 x 12 diallel was attributed to non-genetic or environmental effects. Therefore, under the conditions described in this study, the genetic control of this trait is fairly weak, and this should be taken into account when interpreting further analysis, for this trait. Analysis of variance of the diallel table, following Hayman (1954), revealed that both additive (a) and dominance (b) effects were significant for shoot regeneration from hypocotyl explants, at the 0.1% level (Table 15). The majority of dominance (b) was accounted for by b_3 (as indicated by the high MS value), which was significant at the 0.1 % level, and is a measure of the residual dominance (*i.e.* the dominance that is not accounted for by directional dominance b_1 and ambidirectional dominance b_2). The significance of b_3 indicates that the control of shoot regeneration from hypocotyl explants may not fit a simple genetic model, as such the results from this analysis should be approached with a degree of caution. Maternal effects (c and d) were not significant.

Table 15 **Analysis of variance of the 12x12 diallel table for shoot regeneration from hypocotyl explants, after Hayman (1954), calculated using Genstat version 5.0**

Item	MS	df	F
a	1.32	11	22.4 ***
b1	0.15	1	2.53 n.s
b2	0.04	11	0.64 n.s
b3	0.20	54	3.31 ***
b	0.17	66	2.85 ***
c	0.07	11	1.15 n.s
d	0.05	55	0.77 n.s
block	0.06	572	
error			

Significance level

*** 0.1 %
n.s = not significant

The relationship between the variance of the F_1 offspring to the recurrent parental line (Vr) and their covariance with the non-recurrent parent (Wr) for shoot regeneration from hypocotyl explants was determined, see Figure 13. The difference in Wr and Vr values calculated for each of the parental lines was fairly small, as such the majority of points on the graph clustered in the middle suggesting the trait to be largely controlled by additive gene effects. The trend-line of Wr Vr, does not cut the line of the limiting parabola at either end, suggesting that of the 12 DH parents in the diallel no completely recessive or completely dominant phenotypes are represented. The graph also shows that the dominance relationship is different from that observed for shoot regeneration from cotyledonary explants (In Figure 8, high shoot regenerating genotypes were associated with the low Wr, Vr values and the low regenerators associated with high Wr, Vr values, with intermediate regenerators lying in between). For regeneration from hypocotyl explants this unidirectional dominance does not exist and a completely random pattern is observed; this supports the data in Table 15, where b_3 was identified as being highly significant. More importantly the slope of the Wr, Vr graph, in Figure 13, is significantly different from 1.0 or unity. Had the environmental effects been

minimal (estimated to account for 66.3 % of the variation), the deviation from unity may have been accounted for by epistatic members of the diallel. However, due to the large environmental effects (as indicated by the two-way analysis), and the results of the Hayman analysis, it was concluded that a simple genetic model (of additive and dominant gene effects) could not be fitted to this data set. Indeed, in Figure 14, the plot of Wr + Vr against the mean parental value clearly shows that there is no increase in dominance as shooting increases, or decreases, ($r = 0.0044$, $P > 0.05$). As such, no further biometrical analysis was carried out for this trait.

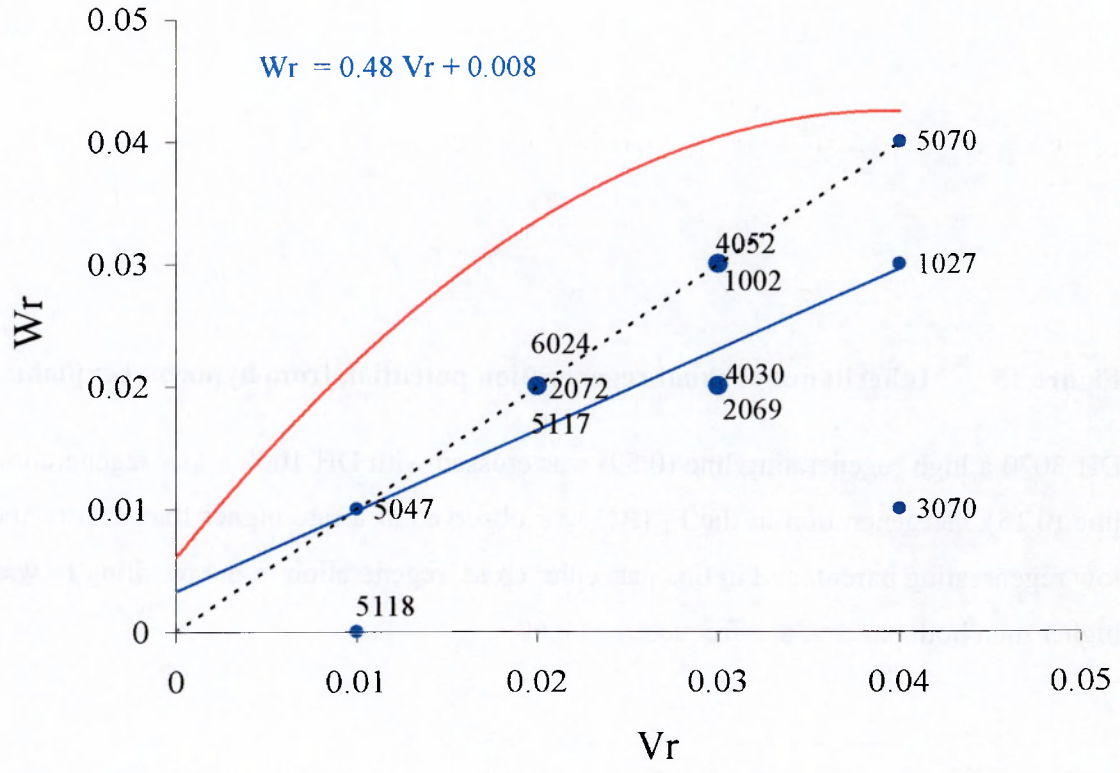
Although the high level of variation that was attributed to non-genetic or environmental effects meant a simple genetic model could not be fitted, it should be noted that shoot regeneration from hypocotyl explants was still shown to be heritable (Figure 15). In comparison to shoot regeneration from cotyledonary petioles, the relationship between genotypes for shoot regeneration, from hypocotyl explants, appears less straightforward. Shoot regeneration from hypocotyl explants (under these conditions) appears to be under a different 'genetic' control than shoot regeneration from cotyledonary petioles (as seen in the DH screen). Two-way analysis of variance carried out on the diallels showed shoot regeneration from hypocotyl explants to be under large environmental, or non-genetic effects (66 %). In comparison, environmental effects only accounted for 18 % of the variation for shoot regeneration from cotyledonary petioles. This difference in environmental errors may account for some of the differences observed, between these two tissue types, for shoot regeneration. The analyses show that additive gene effects account for the majority of the variation observed for shoot regeneration, from both tissue types. However, for shoot regeneration from cotyledonary petioles, dominance gene effects also appear to play a role.

Figure 13 **The relationship between the variance of the F_1, for each parental line (Vr) and their covariance with the recurrent parent (Wr) for shoot regeneration from hypocotyl explants.**

● Plot of Vr vs Wr. The red line is that of the limiting parabola = $\sqrt{Vr.Vp}$ (where Vp is the variance of the parents). The dashed line indicates the theoretical line of unity.

Figure 14 **Wr + Vr from each array of the 12 x 12 diallel plotted against the mean of the common parent, for shoot regeneration from hypocotyl explants.**

No direct relationship appears to exist between increasing dominance (Wr + Vr axis) and the increase in trait (increase in mean parental value for shoot regeneration from hypocotyl explants, x-axis).


Wr = 0.48 Vr + 0.008
0.05
0.04
0.03
0.02
0.01
0
Wr
0
0.01
0.02
0.03
0.04
0.05
Vr
5070
4052
1002
1027
6024
2072
5117
4030
2069
5047
3070
5118

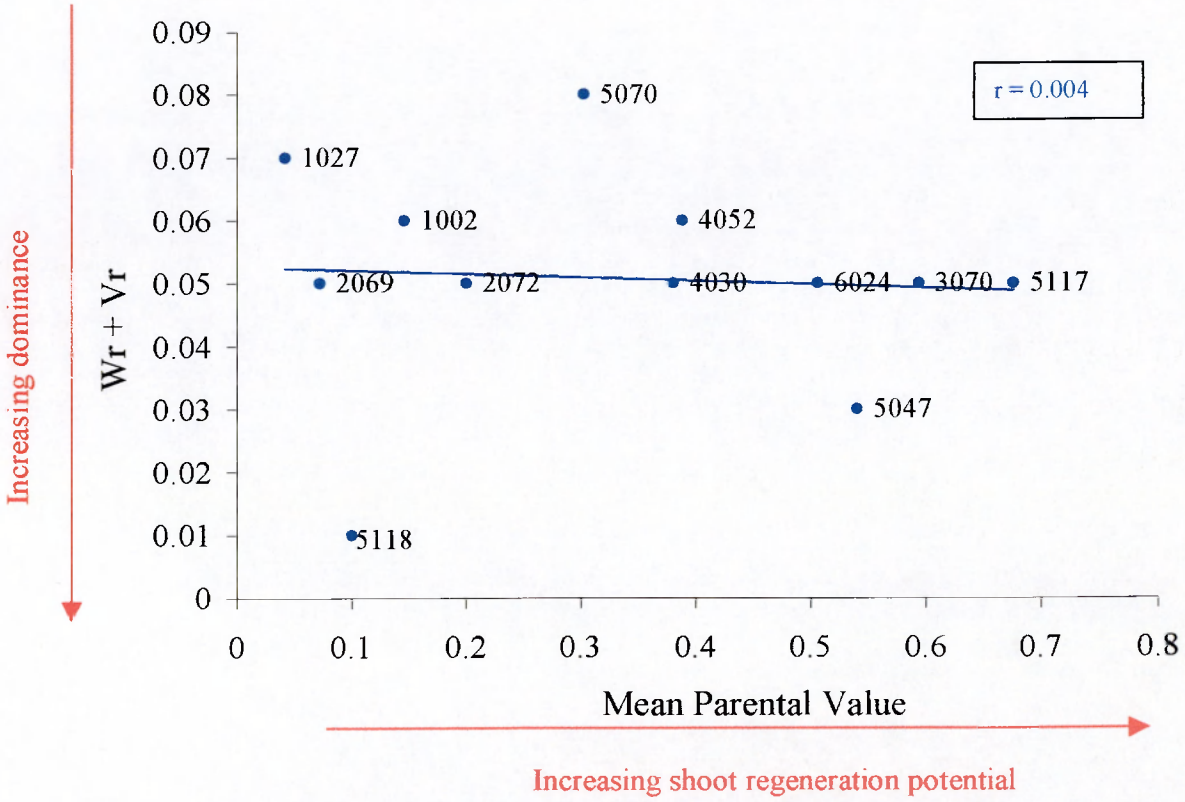
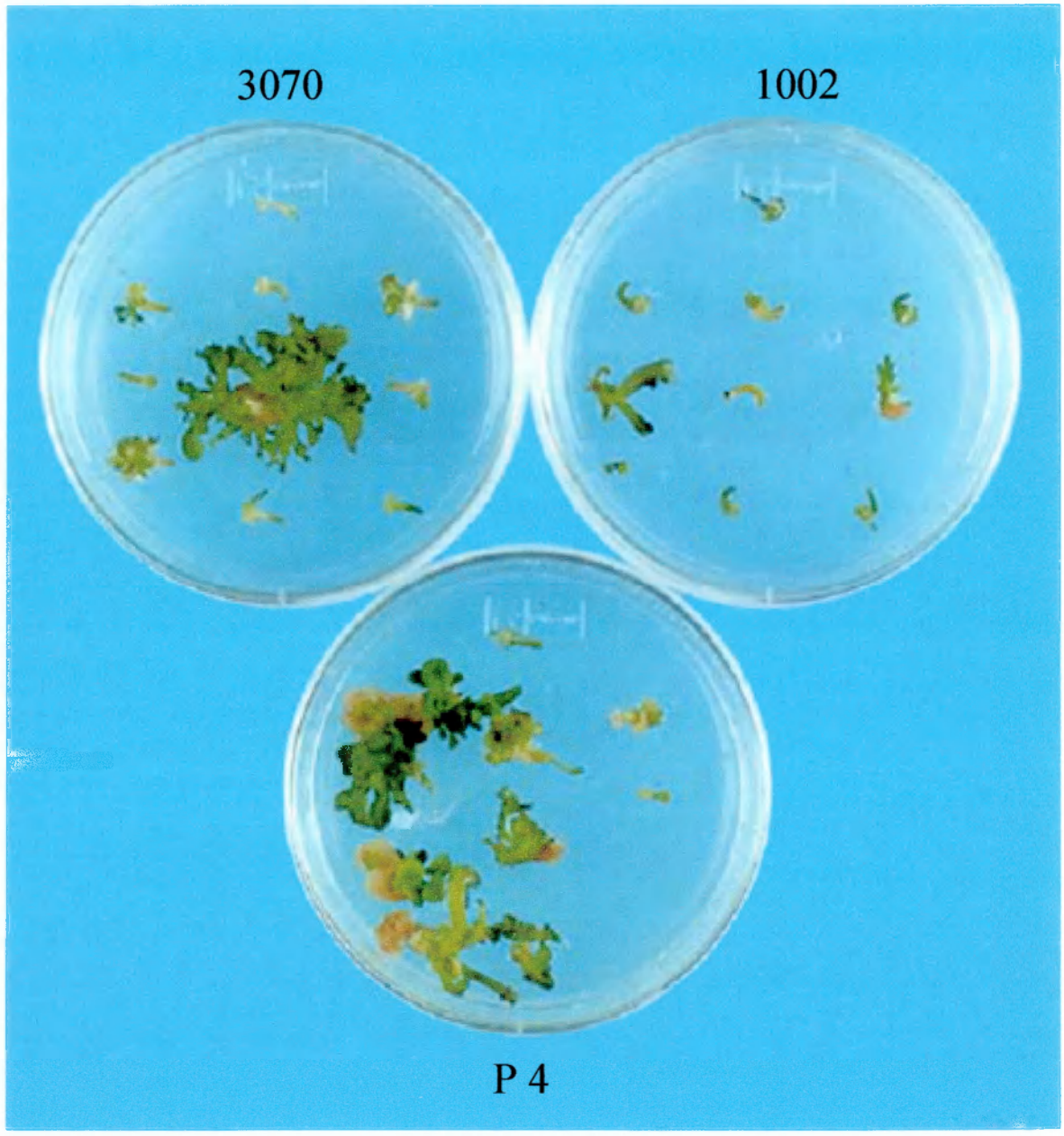

r = 0.004
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Wr + Vr
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Mean Parental Value
5070
1027
1002
4052
2069
2072
4030
6024
3070
5117
5047
5118
Increasing dominance
Increasing shoot regeneration potential

Figure 15 Inheritance of shoot regeneration potential, from hypocotyl explants.

DH 3070 a high regenerating line (0.59) was crossed with DH 1002 a low regenerating line (0.15). Regeneration in the F_1 (P4) was observed at a rate higher than that of the low regenerating parent, and in this particular cross, regeneration in the resulting F_1 was higher than both parents, at a frequency of 0.60.


3070
1002
P 4

3.3.3.4 Root regeneration from cotyledonary explants

The 12 DH parental lines and 128 F_1 hybrids were scored for the presence or absence of roots, regenerating from the cotyledonary petiole base, after 44 days in culture. Root regeneration rates (expressed as mean counts) for the F_1 and 12 DH lines are presented in Table 16. The red diagonal line on the diallel table shows the root regeneration responses of the 12 DH parents. When compared to Table 8 (the diallel table for shooting from cotyledons) it is clear that the order of the DH parents for root regeneration potential (from high to low) is different from that of shoot regeneration (from high to low). No correlation ($r = -0.02$, $P > 0.05$) was observed when the data from the two diallel tables were compared (for shoot and root regeneration). Therefore, there appears to be no direct relationship between shoot and root regeneration potential.

Table 16 Diallel table of root regeneration (expressed as mean counts) from cotyledonary petioles, after 44 days in culture.

Male / Female	5070	3070	5047	5117	4052	6024	4030	2072	5118	2069	1027	1002
5070	**0.04**	0.05	0.00	0.70	0.27	0.11	0.25	0.11	0.25	0.11	0.04	0.20
3070	0.00	**0.13**	0.09	0.44	0.59	0.26	0.63	0.00	1.00	0.00	0.14	0.26
5047	0.00	0.09	**0.01**	0.36	0.13	0.16	0.17	0.24	0.17	0.04	0.04	0.06
5117	0.40	0.47	0.36	**0.65**	0.60	0.50	0.80	0.61	0.58	0.17	0.35	0.63
4052	0.21	0.59	0.23	0.60	**0.24**	0.43	0.55	0.58	0.65	0.22	0.09	0.30
6024	0.11	0.26	0.10	0.60	0.72	**0.24**	0.48	0.48	0.55	0.17	0.06	0.52
4030	0.37	0.58	0.18	0.80	0.63	0.45	**0.53**	0.69	0.82	0.25	0.12	0.61
2072	0.11	0.10	0.29	0.75	0.80	0.53	0.74	**0.48**	0.46	0.28	0.23	0.45
5118	0.25	1.00	0.17	0.58	0.65	0.55	0.82	0.46	**0.81**	0.23	0.24	0.43
2069	0.17	0.00	0.16	0.22	0.49	0.17	0.35	0.38	0.15	**0.02**	0.06	0.27
1027	0.04	0.14	0.24	0.55	0.35	0.22	0.28	0.23	0.24	0.06	**0.02**	0.13
1002	0.20	0.26	0.10	0.63	0.59	0.42	0.51	0.45	0.43	0.27	0.13	**0.31**

Where no F_1 seed was available for analysis, the reciprocal F_1 value was used instead (indicated by numbers in blue). Parental values are given in red.

Two-way ANOVA on this data set indicated that 30 % of the variation observed between genotypes in the 12 x 12 diallel could be attributed to non-genetic or

environmental effects. Therefore, for root regeneration, 70 % of the variation was attributed to genetic effects. The ability to regenerate roots was also observed to be a heritable trait. Crossing a low root regenerating line with a higher regenerating line often resulted in a regeneration response higher than that of the lower regenerating parent. Analysis of variance of the diallel table, following Hayman (1954), revealed that both additive (a) and dominant (b) effects were significant for root regeneration from cotyledonary petioles, at the 0.1% level (Table 17). Additive effects were far more important, accounting for a high proportion of the total MS. The majority of dominance (b) was accounted for by b_3, which was significant at the 0.1 % level, and is a measure of the residual dominance (*i.e.* the dominance that is not accounted for by directional dominance b_1 and ambidirectional dominance b_2). The significance of b_3 indicates that the control of root regeneration from cotyledonary petioles may not fit a simple genetic model. The average maternal effect (c) was significant at the 1.0 % level but (d), the measure of variation between reciprocals not attributed to specific maternal effects, was non-significant. This suggests that even though reciprocal values were used to replace any missing values in the diallel table (indicated by blue text in the diallel table), the ANOVA was still able to detect maternal effects in this data set.

Table 17 **Analysis of variance of the 12 x 12 diallel table for root regeneration from cotyledonary petioles, after Hayman (1954), calculated using Genstat version 5.0**

Item	MS	df	F
a	2.637	11	110 ***
b1	0.165	1	6.86 *
b2	0.085	11	3.5 **
b3	0.172	54	7.18 ***
b	0.158	66	6.6 ***
c	0.085	11	3.5 **
d	0.017	55	0.70 n.s
block error	0.024	572	

Significance level
* 5 .0 %,
** 1.0 %
*** 0.1 %
n.s = not significant

The relationship between the variance of the F_1 offspring to the recurrent parental line (Vr) and their covariance with the non-recurrent parent (Wr) for root regeneration from cotyledonary petioles is shown in Figure 16. The low root regenerating lines (DH 5047, 2069 and 1027) had the smallest Vr and Wr values and this indicates that the ability to regenerate roots at a low frequency may be dominant over high root regeneration ability (*i.e.* root regeneration is controlled by recessive genes). However, the slope of the Wr, Vr regression line was significantly different from 1.0 (slope = 0.48). As it is a prerequisite for the interpretation of these graphs that the slope should not be significantly different from 1.0 (Jana, 1975), the data from the diallel table, in its current form, did not fit a simple additive-dominance genetic model. Certain types of epistasis can distort the Wr, Vr graph in characteristic ways, and thus permit their detection (Hayman, 1957; Mather, 1967; Coughtrey and Mather, 1970). The departure from the line of unity can potentially be corrected by omitting the arrays corresponding to the questionable and presumably epistatic parents. A useful genetic interpretation of the Wr, Vr graph calculated from the remaining arrays can then be made on the basis of the additive-dominance model. In Figure 16 a theoretical slope of 1.0 was also plotted. The degree to which the Wr, Vr slope (of 0.48) deviates from the line of unity can be seen. Two DH parental lines (DH 5118 and 3070) were shown to deviate the most from the line of unity and both arrays were considered for removal. Prior to this the relationship between Wr + Vr against the mean of the common parent was determined, see Figure 17. As the mean parental value rises within the DH parental arrays, so too did the Wr + Vr value associated with that array, with the exception of DH 3070. This line had a relatively small mean parental value but the largest Wr + Vr value, showing that the means of the F_1 hybrids associated with this array differed considerably from the mean of DH 3070. The removal of array DH 3070 from the diallel produced a Wr, Vr graph with a slope of 0.86, Figure 18. This value was not significantly different

from 1.0 and further analysis on the assumption of an additive-dominance model was carried out, on the 11 x 11 diallel (*i.e.* after the removal of the DH 3070 array). The removal of this array also resulted in an increased correlation (0.6) between Wr+Vr and the mean parental value (Figure 19), but still not all points lie close to the regression line, and again this suggests that the association with increasing dominance and decreasing root regeneration potential does not hold true for all parents.

ANOVA on the resulting 11 x 11 diallel, according to Hayman (1954), gave results similar to Table 17 with the exception that the significance of b_2 rose from P = 0.01 to 0.001 (data not shown). The genetic component analysis for root regeneration, as determined using data from the 11 x 11 diallel, is presented in Table 18. The additive genetic effect (D) was again shown to be more important than dominant effects (H_1 and H_2) which were calculated as zero, using this analysis. This suggests rooting to be controlled entirely by additive gene effects.

Table 18 **Genetic Component Analysis: Root regeneration from cotyledonary petioles.**

Component	11 x 11
D	0.0537
H_1	0
H_2	0
E	0.0247
Mean degree of dominance $\sqrt{H_1/D}$	-
Proportion of dominance $H_2/4H_1$	-
Broad-sense heritability	0.64
Narrow-sense heritability	0.64

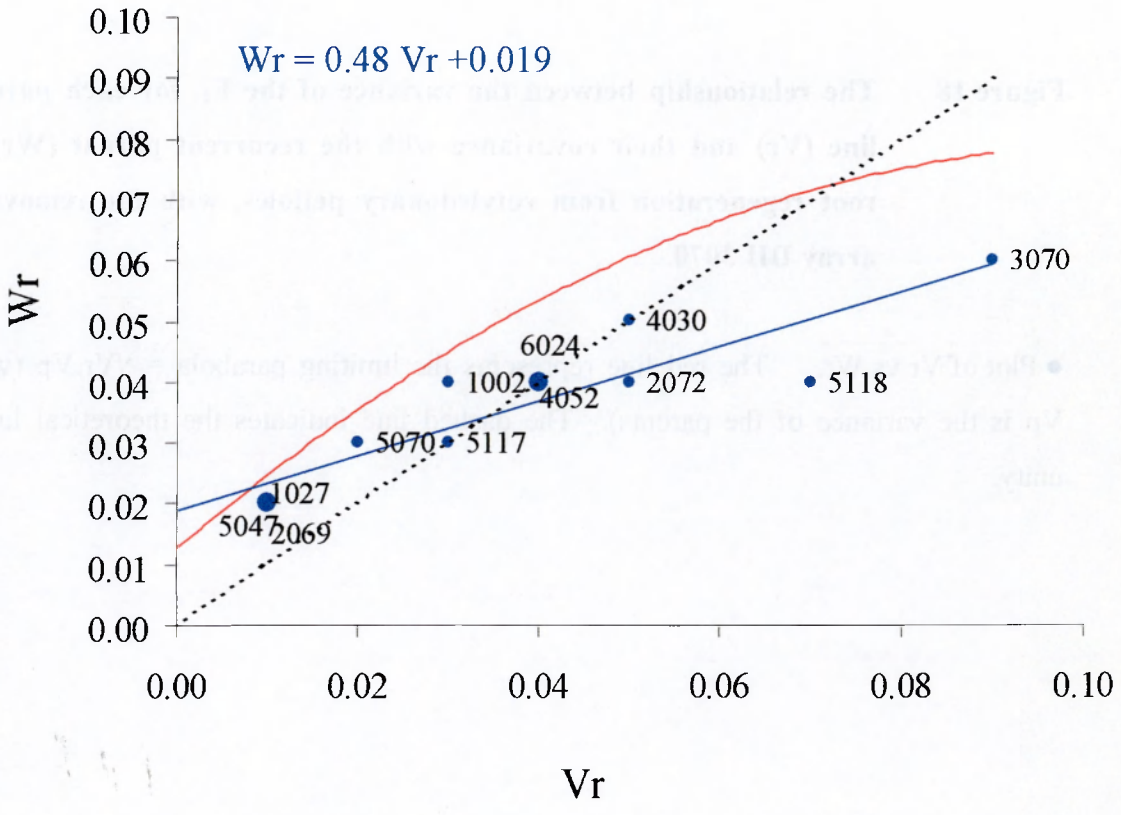
The broad-sense heritability value suggests approximately 64 % of the variation observed within the diallel is genetically controlled, with approximately 36 % of the variation a result of environmental or non-heritable effects. This value is not significantly different from than that calculated by Two-way ANOVA (30 %), for the

12 x 12 diallel). The narrow-sense heritability suggests 64 %, and therefore all of the genetic variation, to be a result of additive gene effects.

Figure 16 **The relationship between the variance of the F_1, for each parental line (Vr) and their covariance with the recurrent parent (Wr) for root regeneration from cotyledonary petioles.**

• Plot of Vr vs Wr. The red line represents the limiting parabola = $\sqrt{Vr.Vp}$ (where Vp is the variance of the parents). The dashed line indicates the theoretical line of unity.

Figure 17 **Wr + Vr from each array of the 12 x 12 diallel plotted against the mean of the common parent, for root regeneration from cotyledonary petioles.**


Wr = 0.48 Vr +0.019
Wr
Vr
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.00
0.02
0.04
0.06
0.08
0.10
3070
4030
6024
1002
4052
2072
5118
5070
5117
1027
5047
2069

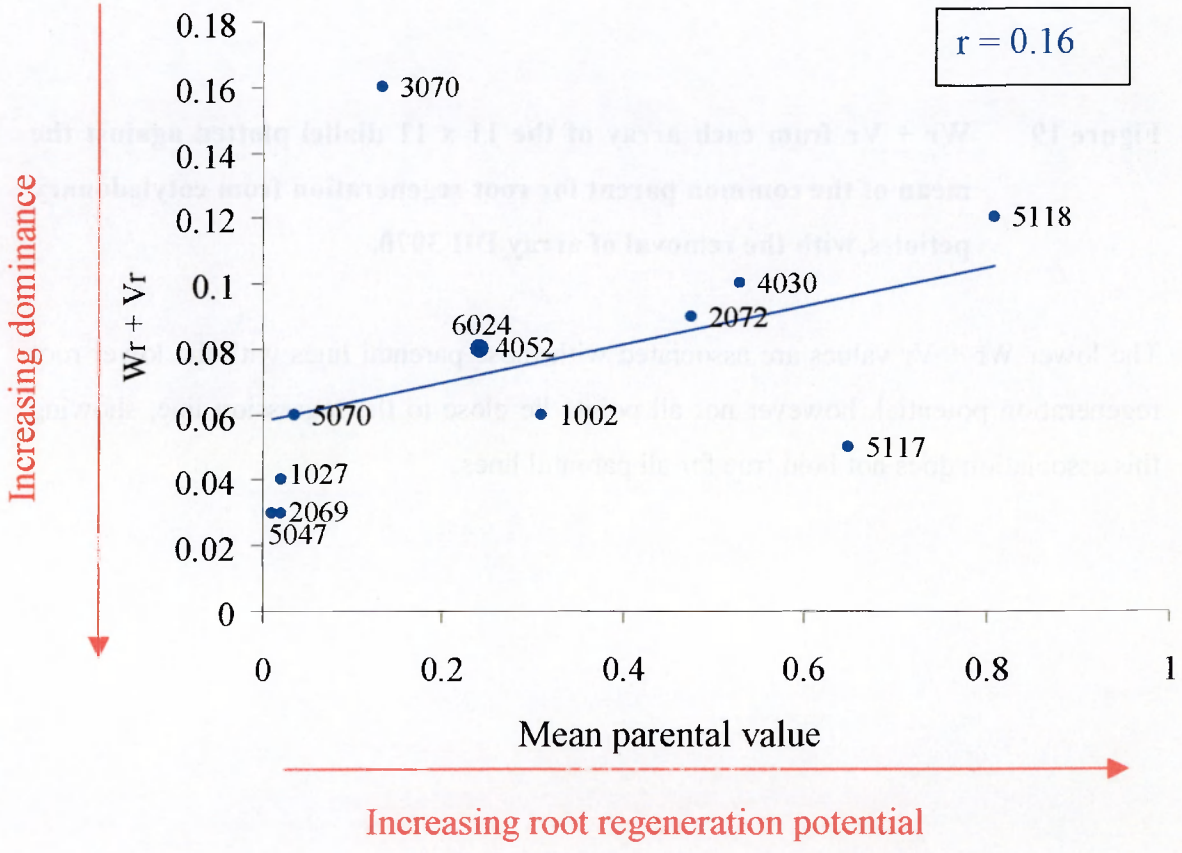
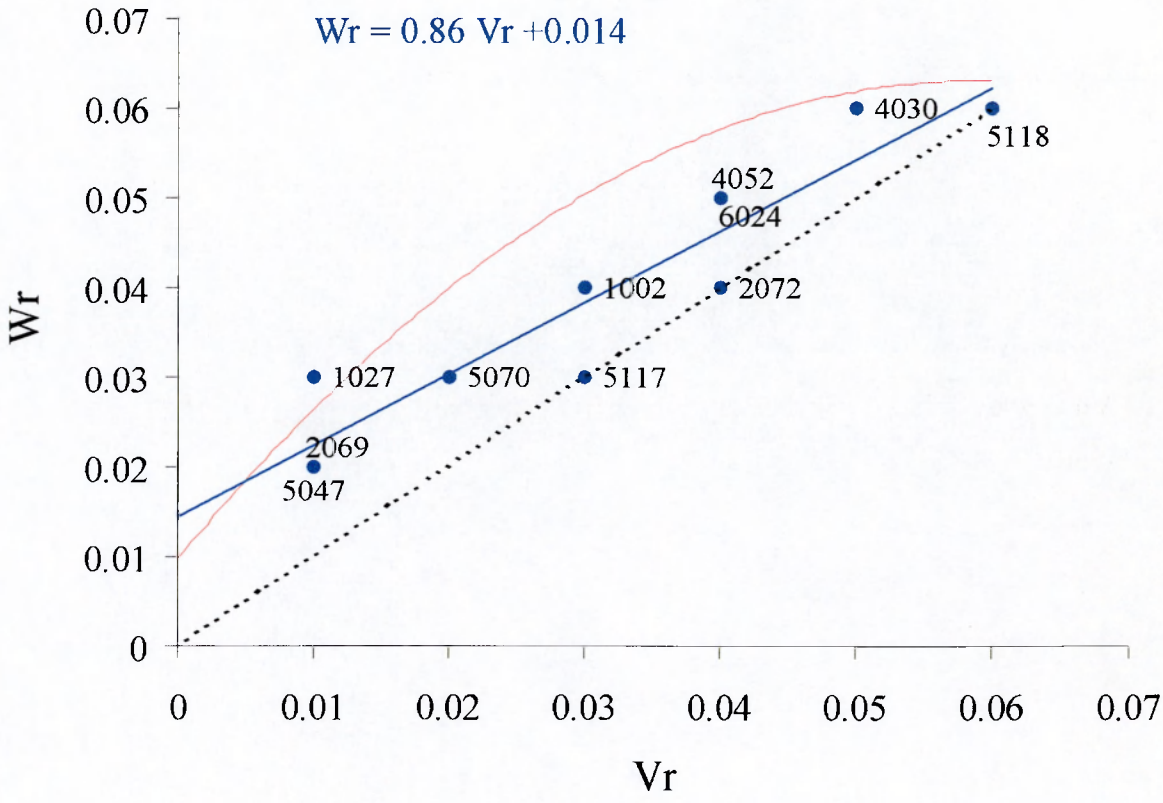

r = 0.16
Wr + Vr
Mean parental value
Increasing dominance
Increasing root regeneration potential
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.2
0.4
0.6
0.8
1
3070
5118
4030
2072
6024
4052
5070
1002
5117
1027
2069
5047

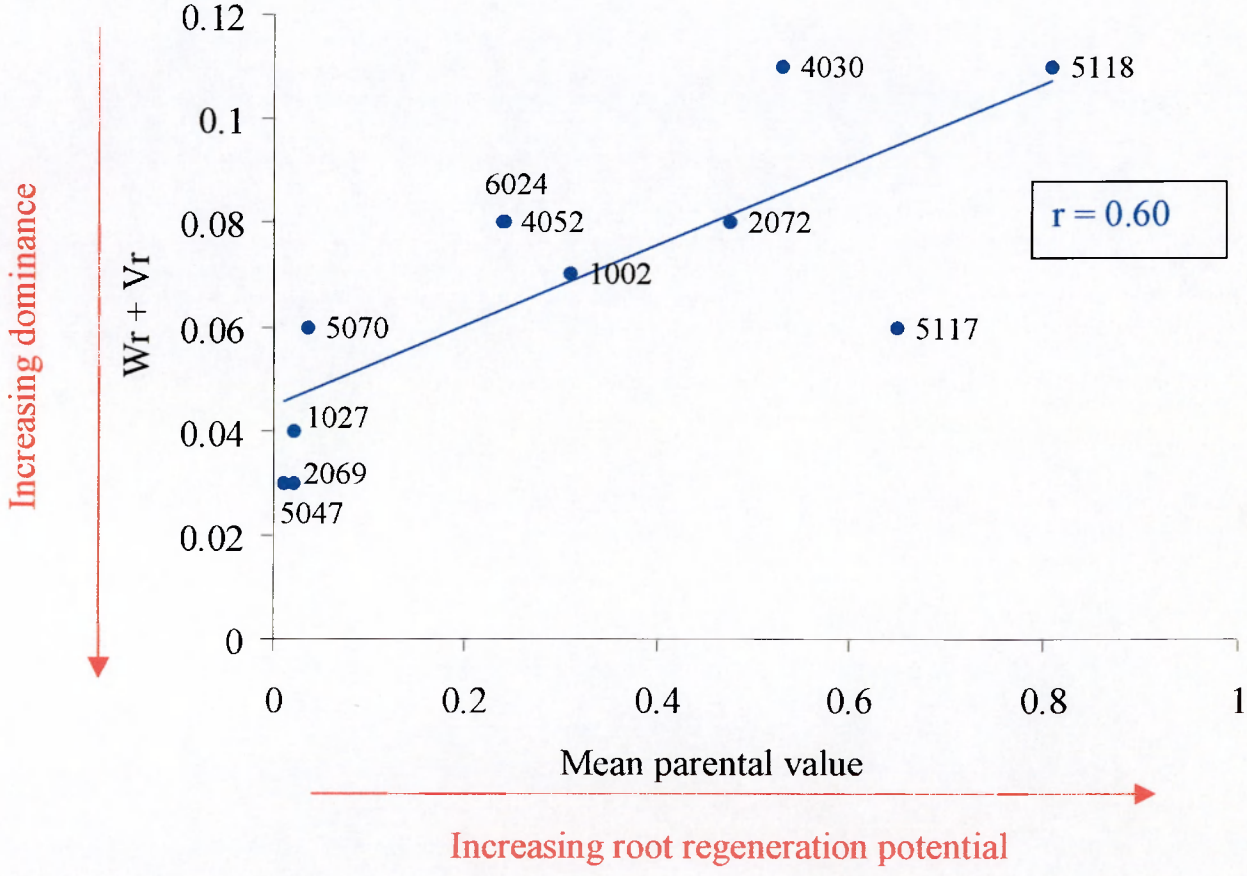
Figure 18 **The relationship between the variance of the F_1, for each parental line (Vr) and their covariance with the recurrent parent (Wr) for root regeneration from cotyledonary petioles, with the removal of array DH 3070.**

• Plot of Vr vs Wr. The red line represents the limiting parabola = $\sqrt{Vr.Vp}$ (where Vp is the variance of the parents). The dashed line indicates the theoretical line of unity.

Figure 19 **Wr + Vr from each array of the 11 x 11 diallel plotted against the mean of the common parent for root regeneration from cotyledonary petioles, with the removal of array DH 3070.**

The lower Wr + Vr values are associated with those parental lines with the lower root regeneration potential, however not all points lie close to the regression line, showing this association does not hold true for all parental lines.


Wr = 0.86 Vr +0.014
Wr
Vr
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
4030
5118
4052
6024
1002
2072
1027
5070
5117
2069
5047


Increasing dominance
Wr + Vr
Mean parental value
Increasing root regeneration potential
r = 0.60
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.2
0.4
0.6
0.8
1
4030
5118
6024
4052
2072
1002
5070
5117
1027
2069
5047

3.3.4 Conclusion from the diallel analyses

Under the experimental conditions described, shoot regeneration from cotyledonary petioles appears to be under strong genetic control with 85 % of the variation accounted for by genetic variation and the remainder a result of non-heritable or environmental influences. The majority of the genetic control was a result of additive gene effects, and high shoot regeneration was observed to be dominant over low shoot regeneration. The production of multiple shoots (in favour of just a few shoots) from regenerating cotyledonary petioles was also shown to be a heritable trait, and additive gene effects accounted for the majority of the variation observed within the diallel. These findings make the potential for introducing shoot regeneration potential into breeding lines a real possibility.

Shoot regeneration from hypocotyl explants again appeared to be controlled mainly by additive gene effects. However, a large proportion of the variation observed for shoot regeneration from hypocotyl explants was attributed to non-genetic or environmental effects (66.3 %), as such, a degree of caution was given to the interpretation of this data set. It was concluded that shooting from hypocotyl explants, under these experimental conditions, did not fit a simple additive-dominance genetic model and so further interpretation on this basis would be unwise.

Additive gene effects were shown to control root regeneration from cotyledonary petioles. Non-genetic or environmental effects accounted for 33 % of the variation observed, which was twice as much as that observed for shoot regeneration from cotyledonary petioles. No relationship was observed between shoot and root regeneration from cotyledonary petioles (correlation coefficient $r = -0.02$, $P > 0.05$). This suggests that the ability to regenerate roots was independent of shoot regeneration, and therefore there was no evidence of antagonistic competition between the two.

The strong presence of additive gene effects for each of these traits suggests the potential for incorporation into breeding programmes.

3.4 The inheritance of shoot regeneration from cotyledonary petioles: Investigating backcrossed and F_2 populations

3.4.1 Introduction

Diallel analysis suggested shoot regeneration from cotyledonary petioles to be controlled by a simple inheritance pattern. The model showed both additive and dominance gene effects to be significant, with additive gene effects accounting for approximately 71 % of the total variation. Such a high degree of additive gene effects suggests the introduction of this trait into subsequent generations to be high. In order to demonstrate the inheritance pattern of shoot regeneration from cotyledonary petioles, a subset of the F_1 (produced in the 12 x 12 diallel) were backcrossed to both their DH parents reciprocally, and also self-pollinated to produce the F_2 population. These populations were then screened for shoot regeneration potential.

3.4.2 Method

Ten families were selected for investigation that contained crosses between lines of different shoot regeneration potentials. The DH parents, backcrossed (BC) and F_2 populations of these families were screened for shoot regeneration response from cotyledonary petioles taken from 4-day-old seedlings as previously described. As the number of BC seed available was a limiting factor, this experiment was set up at the same time as the *A. tumefaciens* susceptibility trials (section 4.6). Cotyledons were removed from 4-day-old seedlings, taking care not to damage the apical meristem, and

the following day the 5-day-old seedlings were inoculated with *Agrobacterium*. Due to the size and complexity of this screen, the experiment was established over four weeks, in the form of four replicates. For the DH parent lines approximately 50 cotyledonary petioles were established in total. For the BC populations approximately 100 cotyledons were established (made up of F_1 x Parent A, Parent A x F_1, F_1' (the reciprocal to F_1) x Parent A and Parent A x F_1') for Parent A and vice versa for Parent B, and approximately 200 cotyledons for the F_2 populations. Due to the difficulty in generating F_1 seed, it was not possible to re-screen the F_1 at the same time as the DH, BC and F_2 populations. The presence or absence of shoots was scored after 44 days in culture.

3.4.3 Prediction of results

The information gained from screening the DH and F_1 lines of the 12 x 12 diallel was used to make predictions on the inheritance of shoot regeneration in the subsequent populations described here. The diallel screen suggested that shoot regeneration response from cotyledonary petioles was predominantly controlled by additive gene effects (71 %). Dominance effects and environmental effects attributed to 9 % and 15 % of the total variation, respectively. High shoot regeneration appeared to be dominant over low shoot regeneration, and the trend generally appeared to be unidirectional. Maternal effects also appeared to be non-significant, and suggests the genetic control to be nuclear rather than cytoplasmic (this enabled reciprocal data to be pooled in this section). Using this information the following predictions were made.

The DH parents of the ten families were screened at the same time as the BC and F_2 populations. The F_1s were not re-screened along side these populations, and therefore the first estimate made was that of the F_1 value. Assuming additive gene

effects to be predominant the F_1 was estimated as the mid-point value between the two DH parents (*i.e.* (Parent A + Parent B)/2). As dominance effects also contribute to the inheritance of this trait, it could be assumed that regeneration rates slightly above the estimated value might be obtained. Likewise, the shoot regeneration response of the BC populations was estimated as the mid-point of the estimated F_1 value and the appropriate DH parent (to which the F_1 had been backcrossed to). Even though more variation (segregation) is observed in a F_2 population, the mean of the population will be the same as the mean of the F_1 population from which it was derived. The estimated F_2 value was therefore the same value as the estimated F_1.

3.4.4 Results

Both the observed and the expected values, for each of the ten families, are presented in Table 19. Crossing two low regenerating phenotypes together (Families 2, 4, 5 and 10) resulted in low shoot regeneration in the subsequent generations (BC and F_2 populations) as expected. In all cases the observed shoot regeneration response was close to that of the estimated value. Any variation from the estimated value was considered not significant, as the number of explants screened and any environmental effects make it hard to precisely estimate the degree of significance on these low regenerating lines.

Inheritance of shoot regeneration potential was observed when a low shoot regenerating phenotype was crossed with an intermediate (Families 1 and 3). In these families however, when the F_1 was backcrossed to the low regenerating parent, shoot regeneration potential, although conserved, was lower than the estimated value. Again the significance of this is hard to estimate, due to the low regeneration response and environmental effects.

Families 6, 7 and 9 (Table 19) represent crosses between low and high shoot regenerating phenotypes. The observed shoot regeneration potentials in Families 7 and 9 were lower than those of the expected values, while in Family 6 they were close to those of the expected values. Crossing a high and an intermediate shoot regenerating line (as Family 8, Table 19) gave observed regeneration rates approximately equal, or slightly higher than, the expected values. The estimated values were made on the assumption of only additive effects being present. Had dominance effects also been a major component of the genetic variation, then values higher than the mid-parent value would have been observed. The fact that values, in general, behaved as expected, and sometimes slightly lower than expected, highlights that additive effects do play the significant role in the genetic control of shoot regeneration.

The data from these families showed that shoot regeneration potential could be passed on to subsequent generations (F_1 and F_2) and that by backcrossing the F_1 to the higher regenerating parent, regeneration rates could be increased significantly.

Table 19: Inheritance of shoot regeneration in backcrossed and F_2 populations.

	Family 1 2072 x 4052		Family 2 1027 x 2069		Family 3 1027 x 4052		Family 4 2072 x 2069		Family 5 2072 x 1027	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
Parent A	0.17	-	0.00	-	0.00	-	0.17	-	0.17	-
Parent B	0.48	-	0.06	-	0.48	-	0.06	-	0.0	-
F_1	-	0.32	-	0.03	-	0.24	-	0.12	-	0.09
BC to Parent A	0.12	0.25	0.06	0.015	0.04	0.12	0.16	0.14	0.09	0.13
BC to parent B	0.42	0.40	0.04	0.045	0.59	0.39	0.13	0.09	0.10	0.04
F_2	0.21	0.32	0.05	0.03	0.21	0.24	0.08	0.12	0.05	0.09

	Family 6 2069 x 5117		Family 7 1027 x 5117		Family 8 3070 x 4052		Family 9 2072 x 3070		Family 10 1002 x 1027	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
Parent A	0.06	-	0.00	-	1.00	-	0.17	-	0.00	-
Parent B	0.89	-	0.89	-	0.48	-	1.00	-	0.00	-
F_1	-	0.48	-	0.45	-	0.74	-	0.59	-	0.00
BC to Parent A	0.27	0.27	0.05	0.23	0.91	0.87	0.11	0.38	0.00	0.00
BC to parent B	0.57	0.69	0.47	0.67	0.88	0.61	0.52	0.80	0.00	0.00
F_2	0.42	0.48	0.16	0.45	0.74	0.74	0.39	0.59	0.01	0.00

3.5 Discussion

A number of populations have been screened for shoot regeneration potential within this study. Variation for shoot regeneration potential was observed in random populations of *B. rapa*, *B. juncea* and *B. oleracea* (Appendix A). Within the DH and F_1 populations, variation for both shooting and rooting ability were observed. These observations confirmed that regeneration was highly genotype dependent and ultimately likely to be under some form of genetic control. A putative QTL was identified for shoot regeneration from cotyledonary petioles on linkage group 01 of the genetic map associated with the DH *B. oleracea* population. The potential significance of this QTL might have been masked by the extreme tissue culture blackening associated with a high proportion of genotypes in this population, and this was believed to have reduced the overall regeneration potential of this population. The random population of *B. oleracea* (Appendix A), and the DH and F_1 population of the diallel cross, contained fewer genotypes that suffered from tissue culture blackening and had higher average shoot regeneration potentials.

The negative effects of tissue culture blackening on the regeneration of transformed shoots have been reported for a number of species for e.g. grape (Colby *et al.*, 1991 and Perl *et al.*, 1996) and soybean (Olhoft *et al.*, 2001 a/b). Tissue browning and necrosis of plant cells are likely defence responses to wounding and / or pathogen infection. When a plant is under attack (from wounding or pathogen infection) one of the first defence mechanisms activated is the production of reactive oxygen species, referred to as oxidative bursts (reviewed by Wojtaszek, 1997). This is thought to activate programmed cell death, or the hypersensitive response, to generate a barrier of dead cells to seal off the site of infection. Upon wounding, dicotyledonous plants also release phenolic compounds (which act as a signal to attract *Agrobacterium*), the

oxidation of these phenolics by polyphenol oxidases (PPOs) and peroxidases (PODs) result in the deposition of tannins and release of phytoalexins, which provide further protection against pathogen infection (reviewed by Vamos-Vigyazo, 1981 and cited in Olhoft *et al.*, 2001 b). The enzymatic production of tannins results in browning of wounded tissues.

In cases associated with this dramatic wound response, wounded cells were often excluded from participating in shoot regeneration, which is unfortunate because wounded cells may be the ones most preferred by *Agrobacterium* for infection (Zambryski, 1988). The reduction of tissue culture browning has been achieved in a number of species by the addition of antioxidants to the culture medium, such as polyvinylpolypyrrolidine (PVP) and dithiothreitol (DTT) (Perl *et al.,* 1996), or agents such as thiol compounds (Olhoft *et al.*, 2001 b). These antioxidants are thought to inhibit the production of PPOs, PODs and enzymatic browning. In these studies the reduction of browning and necrosis of the cells, was associated with an increase in transformation and regeneration events.

In this present study, tissue culture blackening was thought to be a result of a reaction between the explant (perhaps associated with chemicals released as part of the plant defence mechanism) and a reaction with the culture medium (refer back to Figure 4 page 51). It would be of further interest to see if the inclusion of antioxidants into the culture medium would have an effect on this extreme tissue culture blackening as it has on the tissue culture browning reported above. The reduction of tissue culture blackening observed within the DH population may result in an overall increase in shoot regeneration response and therefore may raise the profile of the putative QTL associated with this trait on linkage group 01 of *B. oleracea.*

Further investigation into the genetic control of shoot and root regeneration was carried out using diallel crosses and subsequent biometrical analysis. Under the experimental conditions described, shoot regeneration from cotyledonary explants appears to be under strong genetic control with 85 % of the variation accounted for by genetic variation and the remainder a result of non-heritable or environmental influences. Both additive and dominant gene effects were significant, and high shoot regeneration was dominant over low shoot regeneration. The majority of the genetic variation (71 %) was associated with additive gene effects. A high correlation ($r = 0.8$, $P < 0.01$) was observed between high shoot regeneration and multiple shoot regeneration. The production of multiple shoots (in favour of just a few shoots) from regenerating cotyledonary petioles was also shown to be a heritable trait, and additive gene effects again accounted for the majority of the variation observed within the diallel (77 %). These findings make the potential for introducing shoot regeneration potential into previously recalcitrant lines a real possibility.

The inheritance of shoot regeneration from hypocotyl explants (Figure 15, page 85) was also observed. However, a large proportion of the variation observed was attributed to non-genetic or environmental effects (66.3 %), and therefore, a degree of caution was given to the interpretation of this data set. It was concluded that shooting from hypocotyl explants, under these experimental conditions, did not fit a simple additive-dominance genetic model, but it was proposed that additive gene effects again played a major role.

Root regeneration from cotyledonary petioles was also shown to be controlled by additive gene effects. Non-genetic or environmental effects accounted for 33 % of the variation observed, which was twice as much as that observed for shoot regeneration from cotyledonary petioles (and as the experimental conditions were selected to favour shoot regeneration, this finding is perhaps not surprising). No relationship was

observed between shoot and root regeneration from cotyledonary petioles (correlation coefficient - 0.02, $P > 0.05$). This suggests that the ability to regenerate roots was independent of shoot regeneration, and therefore there should be no antagonistic competition between the two.

The strong presence of additive gene effects for each of these traits suggests the potential for their incorporation into economically important cultivars showing low or unresponsive tissue culture qualities.

How does the information gained in this study help to explain what has previously been reported in the literature? In Chapter 1 section 1.4.1.1 the variation for shoot regeneration, both between and within the *Brassica* species, was discussed and included an assessment of evidence that shoot regeneration is potentially under genetic control. The literature discussed gave evidence that *B. rapa* (the AA genome) was the most recalcitrant of the *Brassica* species for *in vitro* shoot regeneration. *B. oleracea* (the CC genome) was reported as being highly amenable to shoot regeneration and *B. napus* (AACC) had an intermediate response between the two genomes (AA and CC), with shoot regeneration ability thought to be inherited from the CC genome. The results presented in Chapter 3 and Appendix A show that even within *B. oleracea* there are lines that are recalcitrant to regeneration. However, the C genome of *B. oleracea* may contain, or have conserved, more high shoot regeneration genes than the A genome (Appendix A (A.2.3)). This was shown clearly in the paper reporting the regeneration response of synthetic *B. napus* (AACC) Narasimhulu *et al.,* (1988 a,b). Crossing a low regenerating *B. rapa* (AA) with a high regenerating *B. oleracea* (CC) resulted in an intermediate response in the AACC genome of *B. napus.* In this current chapter (section 3.3.3.1) it was reported that crossing a low shoot regenerating line with a high, would result in an intermediate response in the F_1. Therefore, compared to the

regeneration response observed in the synthetic *B. napus*, it would appear that crossing genomes of differing shoot regeneration response can have the same result as crossing two genotypes, from the same species, of differing shoot regeneration response. The finding in the literature that there is no strong evidence for whether the BB genome is better at regenerating than the CC genome, would suggest that both genomes have high conservation of genes associated with high shoot regeneration. This idea is supported by the findings of Ono *et al.*, (2000) who looked at the genetic control of shoot regeneration in *B. napus*. In this paper the inheritance of shoot regeneration was investigated using a 5 x 5 diallel cross, which included genotypes with a range of shoot regeneration potentials (from 0 to 96.7%). As with the data presented in section 3.3.3.1, they found that shoot regeneration from cotyledonary petioles was associated with additive and dominant gene effects. Dominant genes had a positive effect on shoot regeneration (*i.e.* high shoot regeneration was dominant over low shoot regeneration). Again dominance of this trait was incomplete, and additive gene effects accounted for the majority of the variation (82 % in this population).

The findings from this present study, in relation to other work in this field, would suggest that the same genes responsible for shoot regeneration have been conserved within the *Brassica* genus.

Chapter 4 Susceptibility to *Agrobacterium tumefaciens*

4.1 Introduction

Agrobacterium tumefaciens is a soil-borne bacterium that causes crown gall disease in a wide range of dicotyledonous plants. Crown galls result from the transfer of T-DNA, containing tumour-inducing genes, from the wild-type bacterium to a susceptible plant cell and its subsequent integration and expression (see also section 1.3.1.1). This infective process is a natural form of genetic engineering. By replacing tumour-inducing genes with genes of interest, the utilisation of *A. tumefaciens* as a delivery system has become common practice. Despite advances in genetic transformation the ability to transform plant species is still hindered by genotype restrictions, with some lines being resistant to infection. It is therefore an important prerequisite when using *A. tumefaciens* as a transformation system, that the plant genotypes selected are susceptible to *A. tumefaciens* and thus competent for transformation.

Genetic variation for *A. tumefaciens* susceptibility has been observed in a wide range of plant species including *Prunus* (Bliss *et al.,* 1999), soybean (Bailey *et al.,* 1994; Mauro *et al.,* 1995) and grape (*Vitis* sp.) (Szegedi and Kozma 1984). The cause of the variation in these species is still unknown, but resistance to *A. tumefaciens* is passed on to subsequent generations through both self- and cross-pollination, confirming the character to be a heritable trait. In grapevine, resistance to *A. tumefaciens* appears to be inherited as a single dominant gene, while in other species e.g. soybean (Bailey *et al.,* 1994; Mauro *et al.,* 1995) susceptibility is dominant and often quantitative. In *Arabidopsis thaliana* (Nam *et al.,* 1997) susceptibility again appeared to be the dominant trait, and in one ecotype segregated as a single major

contributing locus (however, the possibility of additional segregating loci contributing to tumorigenesis, could not be ruled out).

For this Chapter susceptibility was not only a measure of a genotypes ability to attract *A. tumefaciens* but also a measure of the uptake, integration and expression of the T-DNA from *A. tumefaciens*. To facilitate a quick visual reference, wild type *A. tumefaciens* strains were used to test for susceptibility. The production of crown galls (the resulting symptom of wild-type T-DNA insertion and expression) was indicative of susceptibility to *A. tumefaciens*. The following populations were screened for susceptibility to three strains of wild-type *A. tumefaciens:*

1. Fifty nine DH lines from the *B. oleracea* mapping population, along with the two parental lines, A12DHd and GDDH33 (detailed in 2.1).
2. Five Substitution lines (of A12DHd genomic backbone with GDDH33 substitutions) described further in section 4.3.2.1.
3. A subset of the F_1 population and DH parents generated in the 12 x 12 diallel programme, (detailed in section 2.1.2).
4. Backcrossed and F_2 populations derived from a subset of F_1 lines, from the diallel programme (detailed in section 2.1.3).

The aim of this Chapter was to identify the genetic loci and inheritance patterns associated with susceptibility to *A. tumefaciens,* using the populations described above. The potential to introduce susceptibility into recalcitrant lines to alleviate genotype restrictions for genetic transformation is discussed.

4.2 Screening of a *B. oleracea* DH mapping population

4.2.1 Method

4.2.1.1 Plant material and genetic map

Fifty nine DH lines from the *B. oleracea* mapping population (detailed in section 2.1) and the two parental lines, A12DHd and GD33DHd were screened for susceptibility to three wild-type strains of *A. tumefaciens.* The associated genetic map used to determine the quantitative trait loci (QTL) was that of Sebastian *et al.,* (2000).

4.2.1.2 *Agrobacterium tumefaciens* strains

Three wild-type strains of *A. tumefaciens* were used in this study; an octopine strain Ach5 and two nopaline strains C58 and T37. The growth conditions for these *A. tumefaciens* strains are detailed in section 2.4.1.

4.2.1.3 Experimental procedure

Five-day-old sterile seedlings were inoculated with *A. tumefaciens* as described in section 2.4.2. For each DH line 25 explants (5 replicates, with 5 seedlings per pot) were established with each of the three *A. tumefaciens* strains. Ten control explants per genotype were also established, with seedlings inoculated with Minimal A liquid medium containing no *A. tumefaciens.* Seedlings were inoculated, just below the meristem, rather than dipping the cotyledonary petioles into a bacterial suspension, as per the transformation protocol, so that the tissue culture blackening observed in the regeneration trials was not mistaken for a hypersensitive response to *Agrobacterium* infection. The site of inoculation was therefore kept clear of the culture medium. All explants were scored for the presence or absence of crown galls 10, 20, 30, 40 and 50

days after infection. The proportion of explants forming crown galls was analysed using generalised linear models (Abeyasekera and Stern, 2001) with logit link and binomial error in the software package Genstat version 5.0®.

4.2.2 Results and Discussion

The DH lines and the two parents were scored for the presence or absence of crown galls after 10, 20, 30, 40 and 50 days. To prevent miss scoring of swollen/sugary injection sites as crown galls, the 50 day score was chosen as the final score date. Genotypes that had not produced crown galls by this time subsequently failed to produce them. Only the results of the 50 day score are presented here (Table 20).

Table 20 Frequency of crown gall formation 50 days post inoculation, with Ach5, T37 or C58.

The data are presented as the mean frequency across replicates (as the total number of explants with crown galls / the total number of explants inoculated).

DH Line	*A. tumefaciens* Strain Ach5	*A. tumefaciens* Strain T37	*A. tumefaciens* Strain C58
GDDH33	0.89	0.95	1.00
A12DHd	0.00	0.00	0.08
1002	0.00	0.00	0.04
1004	0.38	0.80	0.76
1011	0.52	0.16	1.00
1012	0.55	0.92	1.00
1016	*	*	1.00
1017	0.00	0.43	0.12
1020	0.20	0.80	0.92
1021	0.10	*	0.90
1027	0.00	0.08	0.16
1035	0.79	0.85	0.91
1036	0.04	0.00	0.00
1039	0.00	0.15	0.08
1042	0.04	0.08	0.05
1049	0.00	*	0.12
1058	0.15	0.17	0.20
2056	0.56	1.00	1.00
2066	0.40	0.95	1.00

DH Line	*A. tumefaciens* Strain Ach5	*A. tumefaciens* Strain T37	*A. tumefaciens* Strain C58
2068	0.10	0.65	0.63
2069	0.00	0.08	0.05
2072	0.54	0.92	0.84
2073	0.88	0.68	0.92
2074	0.00	*	*
2075	0.72	1.00	0.92
2134	0.80	1.00	1.00
2190	0.82	1.00	1.00
2208	0.16	1.00	1.00
2270	0.92	1.00	1.00
2221	0.00	0.00	0.04
3013	0.05	0.20	0.19
3066	0.40	0.90	0.80
3070	*	0.05	0.13
3078	0.75	1.00	0.88
3079	0.00	0.08	0.00
3083	0.00	*	0.00
3088	0.18	0.48	0.46
3130	*	0.00	0.10
3235	0.92	1.00	0.96
4030	0.88	1.00	1.00
4031	0.44	0.68	0.56
4034	0.88	0.90	1.00
4052	0.70	0.88	1.00
4054	0.66	1.00	1.00
4137	0.88	1.00	1.00
4199	0.00	0.08	0.12
4201	0.00	0.96	1.00
5005	0.08	0.53	0.84
5047	0.15	0.00	0.15
5075	0.32	0.92	0.96
5076	0.70	*	0.75
5079	0.41	0.60	0.80
5080	0.31	0.78	0.86
5117	0.69	1.00	0.88
5118	0.65	0.96	1.00
5145	0.60	0.75	0.72
5147	0.00	*	0.15
6015	0.00	0.18	0.20
6024	0.41	1.00	0.96
6036	0.00	0.40	0.32
6105	0.00	*	0.00

** = Data unavailable (either due to loss of explants through contamination, or genotypes not screened).*

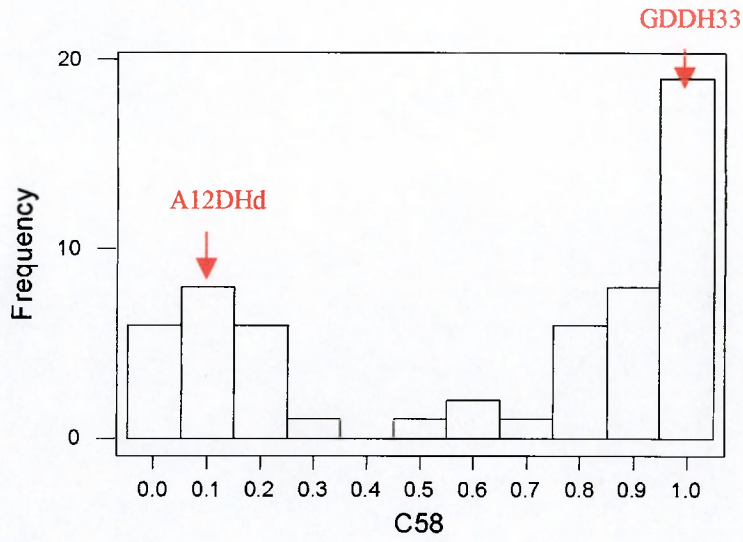
There was a significant difference in the frequency of crown gall production between the two parental lines. GDDH33 was by far the most susceptible parental line to crown gall formation, with nearly all infected explants producing crown galls. This

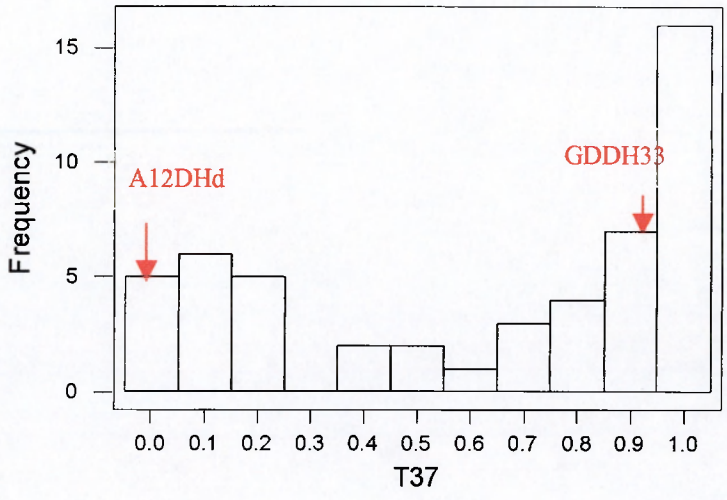
high level of susceptibility was consistently observed in GDDH33 when infected with all three of the *A. tumefaciens* strains (Table 20). There was little difference noted between the three strains for crown gall formation in GDDH33, although the frequency of crown gall formation was slightly less in Ach5, than T37 and highest when infected with C58, statistically there was no significant difference between the three strains. In contrast A12DHd was much less susceptible and failed to produce crown galls when infected with both the octopine strain Ach5 and the nopaline strain T37. Crown galls were observed when A12DHd was infected with C58 but the frequency was low at 0.08 (*i.e.* 2/25 of explants showed crown gall formation). The distribution frequencies for crown gall formation across the DH population fell into similar classes of low and high gall formation, Figure 20. One-way analysis of variance showed there to be no significant difference between replicates within lines, but a significant difference in crown gall formation was observed between DH lines, across the population ($p < 0.001$) when inoculated with each of the three strains, showing susceptibility to *A. tumefaciens* to be genotype dependent.

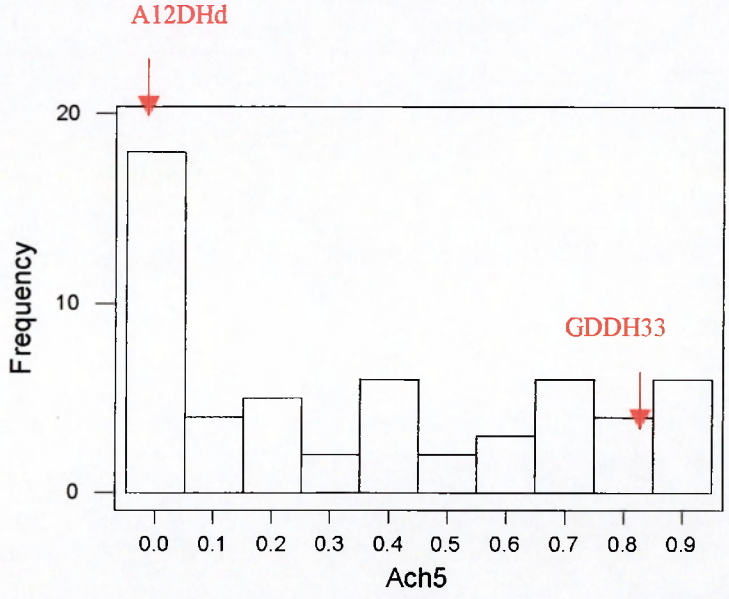
The overall mean frequencies of crown gall formation, across the DH population (Figure 20) were as follows: Ach5 (0.38), T37 (0.61) and C58 (0.63). The distribution frequencies of crown gall formation across the DH population were not statistically significantly different for strains T37 and C58 (Figure 20), with a high correlation coefficient of 0.92 observed between the two strains. T37 and C58 are both nopaline strains, which are reportedly the most virulent of *A. tumefaciens* strains (Fry *et al.* 1987), so it is not surprising that two such strains should both give similar responses.

Figure 20 **Distribution frequencies for crown gall formation across the DH population, 50 days post inoculation with C58, T37 or Ach5.**

The data are presented as counts (i.e. the number of explants forming crown galls / the total number of explants inoculated). The relative position of the two parental lines are indicated in red.


GDDH33
A12DHd
Frequency
20
10
0
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
C58


A12DHd
GDDH33
Frequency
15
10
5
0
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
T37


A12DHd
GDDH33
Frequency
20
10
0
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Ach5

The distribution patterns for the two nopaline strains show crown gall formation, across the DH population, fell into classes of low and high crown gall formation similar to that of the two parental lines. A proportion of the DH lines also showed an intermediate response to crown gall formation, so genotypes could be classified as showing low, intermediate or high crown gall formation when infected with T37 or C58. This kind of distribution pattern (with 'defined' classes) would suggest that susceptibility to *A. tumefaciens* has a relatively simple inheritance pattern.

The distribution frequencies observed when explants were inoculated with Ach5 showed less definition (*i.e.* did not fall into discrete groups) between the number of lines showing low and high crown gall formation. A higher proportion of DH lines failed to produce crown galls and fewer lines showed complete susceptibility to this strain of *A. tumefaciens* (Table 20). The majority of DH lines that exhibited a high frequency of crown gall formation with T37 and C58 also formed crown galls when infected with Ach5, although the frequency of gall formation was generally lower. DH lines that formed crown galls at an intermediate frequency, when inoculated with T37 and C58, often showed low crown gall formation when infected with Ach5, and those DH lines that showed low susceptibility to T37 and C58 also showed low susceptibility to Ach5. This clearly demonstrated that the octopine strain Ach5 was less virulent than the two nopaline strains T37 and C58, supporting the literature that octopine strains are less virulent than nopaline strains. Although the distribution frequencies observed for Ach5 were different, there was still a reasonable association between Ach5 and the two nopaline strains T37 and C58 (correlation coefficients of 0.75 and 0.78 respectively, $P < 0.01$).

To identify the genetic loci involved in the control of susceptibility to *A. tumefaciens,* the data presented in Table 20 (frequency of crown gall formation for each of the three strains of *A. tumefaciens*) was entered into MapQTL®. The response of the

DH lines to *A. tumefaciens* was then compared against the genetic map of this population. On linkage group O9 a large QTL was associated with susceptibility to each of the three *A. tumefaciens* strains, with highly significant LOD scores being observed (peaks of 9, 13 and 24 for Ach5, T37 and C58 respectively). The QTL profiles associated with susceptibility to the three strains of *A. tumefaciens* are shown in Figure 21.

The three profiles show that not only do the two nopaline strains (T37 and C58) share the same QTL profile, but they also coincide with the QTL profile associated with susceptibility to the octopine *A. tumefaciens* strain Ach5. The confidence intervals for these QTL are very large with LOD scores of 3.0 or above covering most of linkage group O9 and, therefore, showing markers along the length of this region to be significantly associated with susceptibility to *Agrobacterium*. All three profiles show the highest LOD score at 40.1 cM along linkage group 09. This position on the genetic map is associated with the RFLP marker pW233.

Single marker analysis was performed using two computer programmes RFLPQB and RFLPRandom to identify the RFLP markers most closely associated with *A. tumefaciens* susceptibility (detailed in section 2.9). Markers that were significantly associated with susceptibility, at the 0.01 % threshold level, were identified on linkage group 09 in the same region as the putative QTL already identified, and can be seen in Table 21. Again the marker pW233 was found to have the highest level of significance, $t = 13.59$ ($P < 0.01$).

Table 21 Single Marker Analysis; to test for significance of markers associated with susceptibility to *A. tumefaciens*, on linkage group 09.

The table presents the LOD scores obtained for each of the markers associated with susceptibility to Ach5, T37 or C58. Markers with LOD scores of above 3.0 (the associated level of significance for QTLs) are shown in blue. t-values (obtained from single marker analysis) are shown for each marker, values that are significant at the 0.01% level are shown in red.

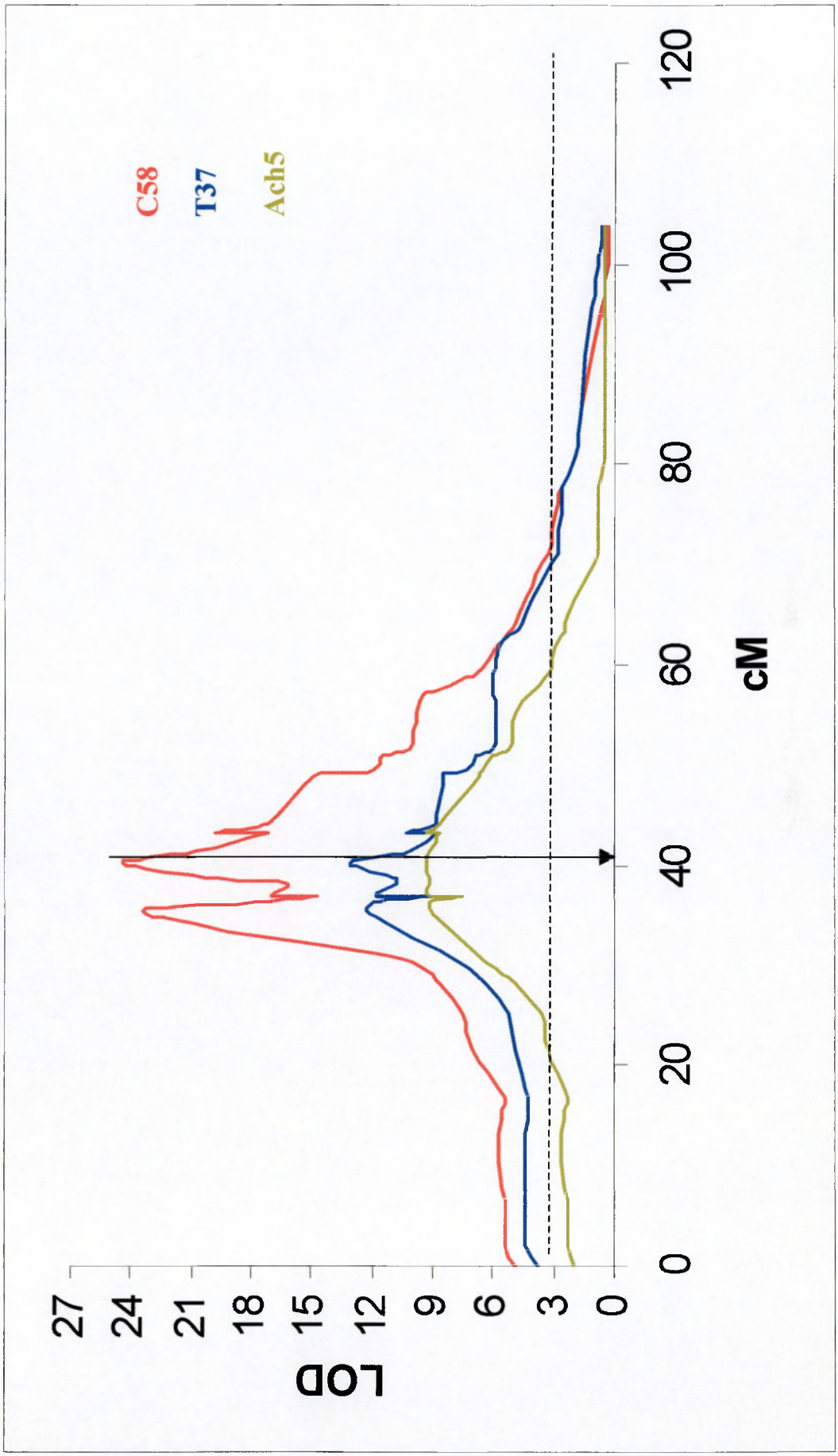
Marker	cM	LOD Ach5	t-value Ach5	LOD T37	t-value T37	LOD C58	t-value C58
pW137J1	0	2.04	2.57	3.81	3.11	4.96	3.73
pW167E2	2.2	2.3	2.54	4.36	3.65	5.46	4.00
pN101E2N	5.5	2.3	2.96	4.37	3.65	5.47	4.36
pW114E2	16.7	2.34	0.35	4.18	1.59	5.41	2.06
pO119J1	25.1	3.6	3.47	5.18	4.48	7.47	5.76
ACCTAE16	28.3	4.9	3.05	6.35	3.86	8.65	4.83
ACCAGJ05	30.2	6.34	4.20	7.7	4.87	10.05	5.86
pCFB8	36.2	9.19	4.02	11.98	9.40	17.23	7.25
ACCATE21	37	9.23	5.82	11.77	8.33	17.08	9.65
pO145E1	38.1	9.22	6.93	10.88	8.09	16.22	9.52
pN173E2	38.7	9.22	6.04	10.88	7.55	19.7	11.63
pW233J1	**40.1**	**9.29**	**7.21**	**13.12**	**8.62**	**24.44**	**13.59**
ACCTCJ01	41.4	9.13	5.92	10.17	6.33	20.75	10.32
pW240E1	43.3	8.7	6.42	8.78	5.33	17.17	8.61
pR34E1	43.3	8.7	4.30	8.78	3.71	17.17	6.65
pN181E1	43.3	9.29	6.62	10.35	6.39	19.86	9.34
pO127E1	43.3	9.29	5.73	10.35	6.65	19.86	10.20
pW135E1	44	8.7	5.58	8.78	6.38	17.17	6.66
pO155E1	44	8.7	5.75	8.78	5.29	17.17	8.64
pR115E1	44	8.7	6.38	8.78	5.33	17.17	8.58
pO168J1	44	8.7	5.48	8.78	5.68	17.17	8.47
ACCAAE08	44	8.7	5.46	8.78	5.93	17.17	9.79
ACCTAJ08	44	8.7	5.82	8.78	6.44	17.17	10.39
pN180E1	49.1	6.68	5.77	8.38	5.90	13.65	8.68
ACCAAE01	49.6	6.54	5.05	7.38	4.85	11.95	7.24
mBN83B1J1	51.1	5.99	5.77	6.83	5.91	11.44	9.79
pW212J1	51.6	5.26	3.89	6.05	3.37	10.07	5.79
pN105E4N	55.2	4.98	4.60	5.9	4.72	9.85	7.05
pW106E1	57.1	4.19	3.35	6.08	4.33	9.55	6.06
pO160E1	57.1	4.19	4.53	6.08	4.85	9.55	7.04
pW195J1	59.2	3.21	3.19	6.08	4.85	6.94	4.90
pW155E1	59.2	3.21	3.34	6.08	4.48	6.94	4.89

Marker	cM	LOD Ach5	t-value Ach5	LOD T37	t-value T37	LOD C58	t-value C58
pO111E2	62.3	2.79	1.77	5.54	1.33	5.5	2.43
pR64E1	63.3	2.52	2.39	4.99	2.25	5.25	2.65
LEW6E2	64.1	2.44	2.80	4.59	4.06	4.9	4.03
pO7E1	71.1	0.84	1.28	2.79	2.51	3.2	2.72
pO118J1	71.1	0.84	1.41	2.79	2.36	3.2	2.64
pC14	77.7	0.77	1.33	2.55	2.85	2.62	2.33
pN47E4NM	80.8	0.52	1.60	1.91	2.57	1.98	2.76
pW200J1	102.6	0.48	1.17	0.7	1.60	0.29	1.71
pN3E1	102.6	0.48	1.01	0.7	1.23	0.29	1.38
pW239E1	103.8	0.48	1.18	0.7	0.57	0.29	1.38

The 0.01 % significance levels were calculated individually for each of the three strains; t-values of above 3.4, 3.5 and 4.3 (for Ach5, T37 and C58 respectively) were significant at the 0.01 % level.

Figure 21 **QTL profiles associated with *A.tumefaciens* susceptibility, on linkage group O9.**

The QTL profiles associated with each of the *A. tumefaciens* strains (C58, T37 and Ach5) had similar coverage across linkage group O9. Although the significance of each strain differed, all were above the level of significance, LOD values $\geq$ 3.0 being significant (Van Ooijen, 1999) as indicated by the dotted line. Each QTL profile hit a peak at 40.1 cM (arrow on figure), and this represented the RFLP marker pW233.


C58
T37
Ach5
LOD
cM
0
3
6
9
12
15
18
21
24
27
0
20
40
60
80
100
120

4.3 Confirmation of QTL position with the aid of substitution lines

4.3.1 Introduction

More precise mapping of QTL can be achieved by specifically selecting for recombination events in particular regions and this essentially requires constructing chromosomes of defined constitution. The basic principle underlying substitution lines is to create genotypes that are identical apart from a defined region on a particular chromosome. Any genetical differences in phenotype, between genotypes, must be due to genes in this defined region. The smaller the region, the more precisely the position of the genes will be known.

A library of recombinant backcross lines (substitution lines) has been produced using the same parental lines as the DH mapping population (Ramsey *et al.*, 1996). The backbone to the substitution lines is that of the A12DHd genome and intergressed into it are varying lengths of the GDDH33 genome. Each unique substitution line has fragments of GDDH33 introduced into one or more of the nine linkage groups of *B. oleracea.* The five substitution lines used in this chapter contain fragments of the GDDH33 genome substituted into linkage group O9, and covering the area to which the QTL associated with *A. tumefaciens* susceptibility has been mapped.

4.3.2 Method

4.3.2.1 Plant material and experimental procedure

Seedlings of A12DHd, GDDH33 and five substitution lines SL141, SL142, SL171, SL175 and SL177 were inoculated with *A. tumefaciens,* strain C58, as previously described. Substitution lines were supplied by Prof. M. Kearsey,

Birmingham University, and seed numbers were bulked up at the John Innes Centre, as described in section 2.1.1. A diagram summarising the genetic make-up of the five substitution lines is shown in Figure 22. Explants were scored after 50 days for the presence or absence of crown galls (see Figure 23).

4.3.3 Results

As expected there was a marked difference in the frequency of crown gall formation between the two parental lines. GDDH33 was highly susceptible to crown gall formation with a frequency of 0.92, while A12DHd remained low with a frequency of 0.04. Substitution of the GDDH33 genome into the beginning or end of linkage group O9, *i.e.* SL172, SL142 or SL175 (see Figure 22), has no effect on introducing or increasing susceptibility to *A. tumefaciens,* as all lines produced crown galls at a rate not significantly different to A12DHd. SL141 and SL177 (see Figure 22) both produced crown galls at a rate similar to GDDH33 (0.92 for both substitution lines), and therefore significantly different to A12DHd. This confirms that the central region of linkage group O9 plays an important role in the genetic control of susceptibility to *A. tumefaciens*, and supports the identification of the QTL located in this region. The high galling lines, SL 141 and SL 177, both shared the same region of GDDH33 genome. It is possible that the genetic region responsible for crown gall formation is contained within this GDDH33 substituted region of 56.5 to 59.6 cM (see footnote[2]), which accounts for 2.7 % of linkage group 09. However, the regions where recombination events have taken place (indicated by the hashed areas in Figure 22) can not be ignored as these areas could contain either of the two genomes. The region of linkage group O9 associated with *A. tumefaciens* susceptibility is therefore identified as being within the

[2] The map lengths associated with the substitution lines can not be directly compared to those of the DH map, as the two populations were generated at different times.

central region, between 24.6 and 67.9 cM, which accounts for 38.5 % of the linkage group. It is suggested, however, that it is within the region between 56.5 and 59.6 cM, that genes responsible for this trait are likely to be. The ability of substitution lines SL 141 and SL 177 to produce crown galls also demonstrates the ability to introduce genes associated with *A. tumefaciens* susceptibility, into otherwise recalcitrant lines.

Figure 22. **Diagrammatic representation of five substitution lines (covering regions of linkage group 09) and the two parental genomes.**

Key:


Represents the A12DHd genome


Represents the GDDH33 genome


Represents regions where recombination events have taken place, and therefore could be of GDDH33 or A12DHd genetic background.

Linkage Group 09

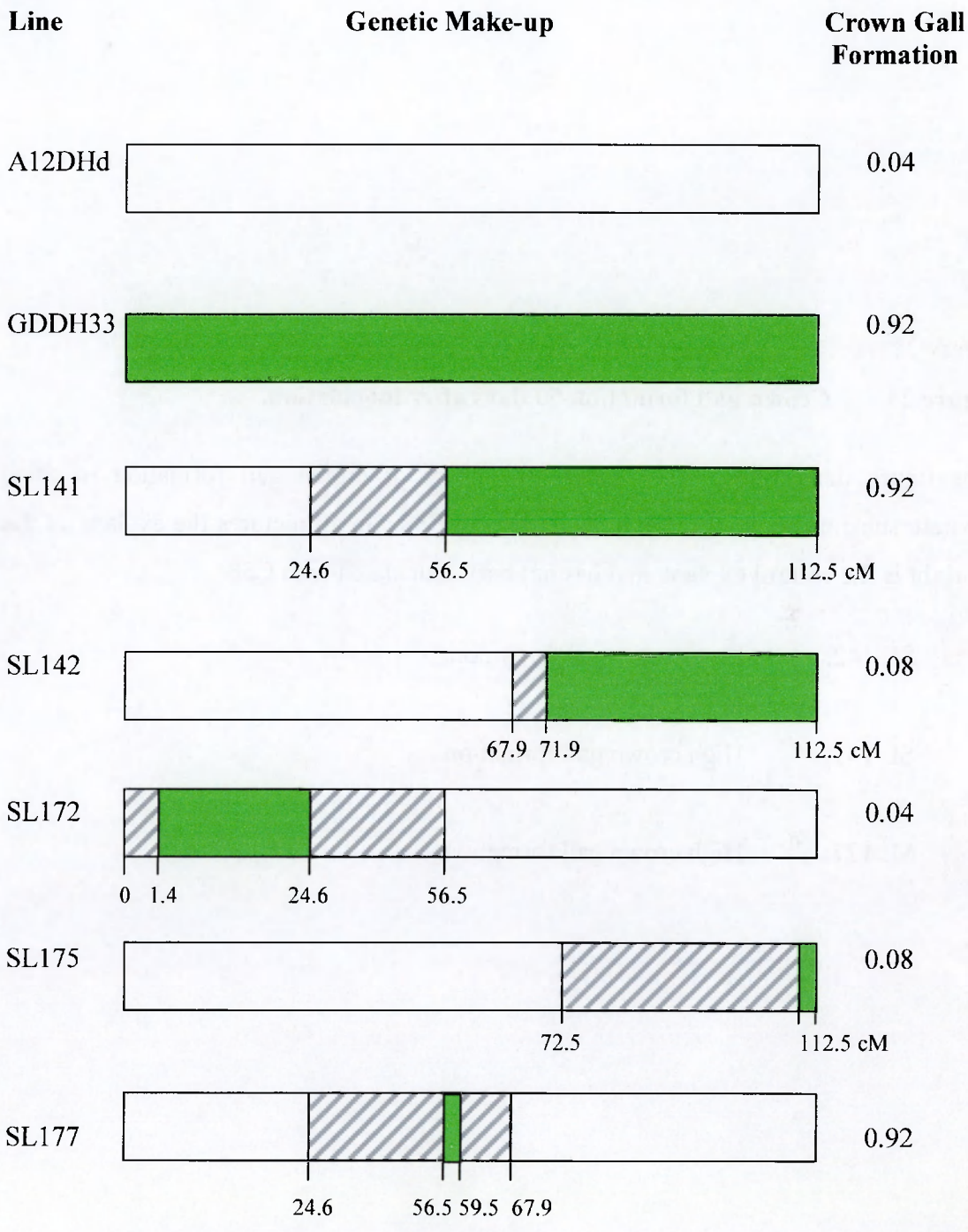


Figure 23 Crown gall formation 50 days after inoculation.

This figure demonstrates the relative frequency of crown gall formation in three separate substitution lines, each inoculated with C58. In all pictures the explant on the far right is the control explant, and has not been inoculated with C58.

a) SL 142: Low crown gall formation.

b) SL 141: High crown gall formation.

c) SL 177: High crown gall formation.

a

b

c

4.4 Testing pW233 as a marker for susceptibility to *A. tumefaciens*

Within *B. oleracea,* susceptibility to *A. tumefaciens* has been shown to be under genetic control, with some genotypes being susceptible and others resistant. The efficiency of *A. tumefaciens* as a transformation system will undoubtedly be affected if the genotype to be transformed is resistant to *A. tumefaciens.* The ability to select lines that are susceptible to *A. tumefaciens* infection may be of advantage to researchers. The potential to use the RFLP probe pW233 as a marker (identified as that most closely associated with this trait) to predict susceptibility to *A. tumefaciens* was investigated.

4.4.1 Method

Southern blot analysis was carried out using the methods described in section 2.8, and using pW233 as an RFLP probe. The Southern blot contained the following genotypes, the two parental lines A12DHd and GDDH33, 10 DH lines from the mapping population (DH 1027, 2069, 5047, 2072, 3088, 4030, 4052, 4031, 6024 and 5117) and the five substitution lines (SL 141, 142, 172, 175 and 177).

4.4.2 Results

A phosphor-image of the probed Southern blot is presented in Figure 24. Polymorphism of the banding pattern (or alleles) associated with pW233 was noted for the two parental lines. DH lines that had previously been identified as high crown gall forming lines (like GDDH33) shared the same banding as GDDH33, while those lines identified as low crown gall formers, shared the banding of A12DHd. Interestingly, those DH lines that produced crown galls at an intermediate level (DH 3088 and 4031)

also shared the banding pattern of A12DHd. This would suggest that lines that showed some degree of resistance to *A. tumefaciens* (observed by lines producing low or intermediate gall formation) share the banding of A12DHd, in this population. The substitution lines that contained the GDDH33 genome within the proposed QTL region were high crown gall formers (SL 141 and SL 177), and both shared the banding pattern for GDDH33. While SL 142, SL 172 and SL 175 (low crown gall formers) contained the A12DHd banding pattern as expected. So pW233 was used to successfully identify genotypes highly susceptible to *A. tumefaciens*, and genotypes with low or intermediate susceptibility to *A. tumefaciens*, although it could not distinguish between the later two.

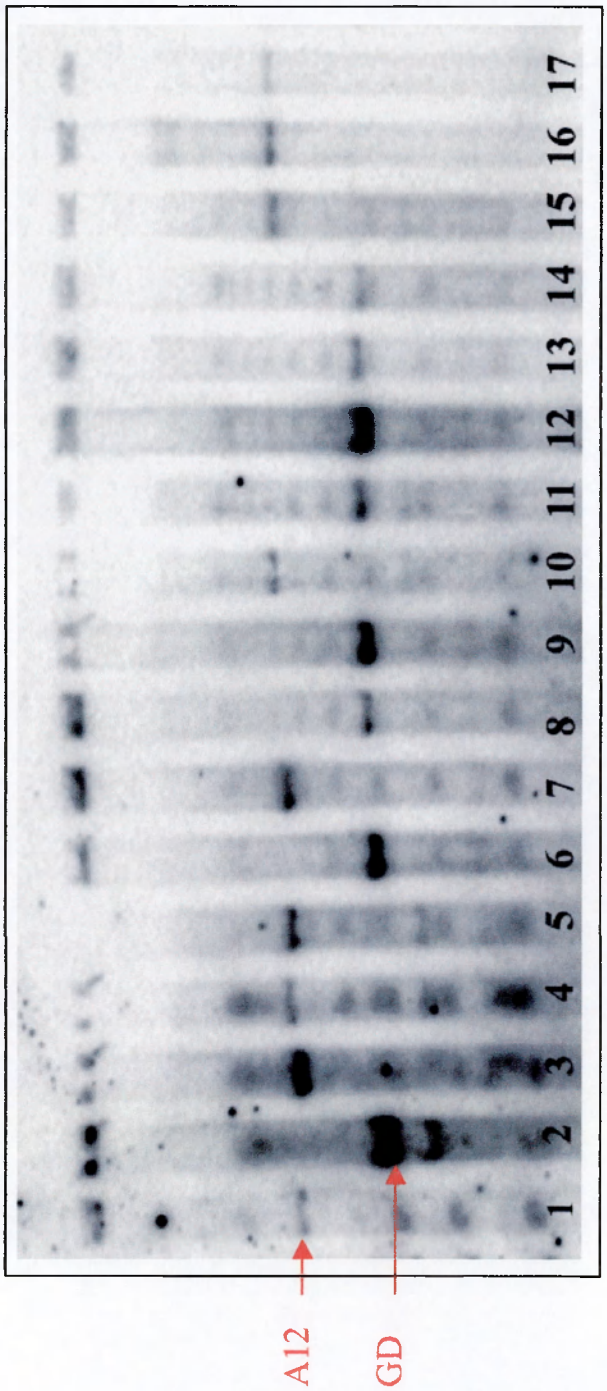
The correlation between genotypes of low, intermediate and high susceptibility to *A. tumefaciens*, and transformation with disarmed strains of *A. tumefaciens* is further investigated in Chapter 5.

Figure 24 Southern blot showing hybridisation of the RFLP probe pW233.

The two parental lines show different banding patterns when probed with this marker (red arrows). Genotypes that form crown galls (CG) at high frequencies share the banding pattern of GDDH33, while low and intermediate crown gall forming lines share the A12DHd banding pattern.

Key:

1. A12DHd (Low CG)
2. GDDH33 (High CG)
3. 1027 (Low CG)
4. 2069 (Low CG)
5. 5047 (Low CG)
6. 2072 (High CG)
7. 3088 (Intermediate CG)
8. 4030 (High CG)
9. 4052 (High CG)
10. 4031 (Intermediate CG)
11. 6024 (High CG)
12. 5117 (High CG)
13. SL 141 (High CG)
14. SL 177 (High CG)
15. SL 142 (Low CG)
16. SL 172 (Low CG)
17. SL 175 (Low CG)


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
A12
GD

4.5 An 8 x 8 diallel analysis: *A. tumefaciens* susceptibility

4.5.1 Introduction

Following the initial screening of the DH population, eight genotypes (chosen from the 12 DH lines that were used to create the original diallel, described in Chapter 3) were selected for diallel analysis. Genotypes were selected to include DH lines that were highly susceptible, and lines that were more resistant to *A. tumefaciens*. The 8 DH lines and 56 F_1 hybrids were used to further investigate the genetic control of *A. tumefaciens* susceptibility.

4.5.2 Method

Eight DH lines (DH 1027, 2069, 5047, 5117, 2072, 4030, 4052 and 6024) and the resulting 56 F_1 hybrid lines were screened for susceptibility to C58 as previously described (section 2.4). The trial was set up over two weeks. Three phytatrays (each with 5 explants inoculated with C58) and one phytatray (containing 5 explants inoculated with liquid Minimal A medium) were established for each of the 64 genotypes, each week. Explants were scored for presence or absence of crown gall formation 50 days after inoculation.

4.5.3 Results

The 8 DH parental lines and the corresponding 56 F_1 lines were scored for the presence or absence of crown galls 50 days after infection with C58. No significant differences were observed between the data collected from the two trials and as such the data has been pooled. The results of these screens are presented in Table 22 as the mean

frequencies (*i.e.* number explants with crown galls/ total number explants infected). Two-way analysis of variance, using a random model, was carried out on the diallel table to determine the amount of variation ascribed to both genetic and environmental effects. The diallel table was analysed using methods described by Hayman (1954) and genetic component analysis was carried out using the methods described by Mather and Jinks (1987). These analyses provide detailed information on the extent and nature of the genetic control of the trait under investigation, and are further described in the relevant sections following.

The diallel contained three DH parental lines that showed low susceptibility (or were resistant) to *A. tumefaciens* (DH 1027, 2069 and 5047) and five lines that showed high susceptibility to *A. tumefaciens* (DH 5117, 2072, 4030, 4052, and 6024). Crossing a DH line with low susceptibility to another low resulted in a low crown gall formation frequency in the resulting F_1. Conversely a high x high cross, resulted in high gall formation frequency in the resulting F_1. The frequency of crown gall formation in a low x high cross was generally much lower than the mid-point of the two parents, suggesting low susceptibility (or resistance) to *A. tumefaciens* to be the dominant trait.

Table 22 8 x 8 diallel table showing crown gall formation 50 days after inoculation with *A. tumefaciens* C58.

The data are presented as frequencies (number explants with crown galls/ total number explants inoculated). Parental values are shown in red.

Female	Male 1	2	3	4	5	6	7	8
1 DH 1027	**0.04**	0.17	0.07	0.04	0.14	0.14	0.00	0.04
2 DH 2069	0.04	**0.10**	0.13	0.10	0.17	0.14	0.11	0.13
3 DH 5047	0.07	0.10	**0.17**	0.26	0.35	0.62	0.41	0.43
4 DH 5117	0.14	0.10	0.26	**0.87**	0.90	0.88	0.94	0.97
5 DH 2072	0.14	0.28	0.12	0.78	**0.72**	0.86	0.97	0.98
6 DH 4030	0.00	0.17	0.60	0.88	1.00	**0.90**	1.00	1.00
7 DH 4052	0.17	0.00	0.34	0.94	0.97	1.00	**0.94**	1.00
8 DH 6024	0.07	0.34	0.39	0.97	0.98	1.00	0.90	**1.00**

Two-way analysis of variance was carried out on the data to estimate the genetic control of this trait. Analysis suggested that just 5 % of the variation, that was observed within the diallel table, was a result of non-genetic or environmental effects, and that 95 % of the variation was due to genetic effects. Further analysis of variance, of the diallel table, following Hayman (1954), revealed that both additive (a) and dominant (b) effects were significant for crown gall formation, at the 0.1 % level (Table 23).

Table 23 Analysis of variance of an 8x8 diallel table for Crown gall formation, after Hayman (1954), calculated using Genstat version 5.0.

Item	MS	df	F	
a	2.2227	7	242.00	***
b1	0.2461	1	26.75	***
b2	0.0546	7	5.9	***
b3	0.1429	20	15.5	***
b	0.1245	28	13.53	***
c	0.0083	7	0.9	n.s
d	0.0092	21	1.0	n.s
block error	0.0092	63		

Significance level

*** 0.1 %
n.s = not significant

Additive effects were by far more important, accounting for the highest proportion of the total MS. The majority of dominance (b) was accounted for by b_1, and shows that dominance is unidirectional. By comparing the mean of the DH parents (0.59) with the mean of the F_1 hybrids (0.46), from the diallel table, low crown gall formation (or resistance to *Agrobacterium*) appeared to show slight dominance over susceptibility to *Agrobacterium*. Maternal effects (c and d) were not significant.

The relationship between the variance of the F_1 offspring to the recurrent parental line (Vr) and their covariance with the non-recurrent parent (Wr) for crown gall formation is shown in Figure 25. The slope of the regression line for Wr, Vr was 0.89, and was not significantly different from a unit slope of 1.0, as such a simple genetic

model of additive-dominant effects for susceptibility towards *A. tumefaciens*, could be fitted. DH lines close to the origin (DH 1027, 2069 and 5047) had the smallest Wr and Vr values, and therefore contained more dominant alleles, while those further from the origin have more recessive alleles. This indicates that low gall formation (or resistance to *A. tumefaciens*) is dominant to high gall formation (or susceptibility to *A. tumefaciens*). The regression line of Wr,Vr intercepted the Wr-axis above the origin, indicating incomplete dominance of the trait. The regression line of Wr, Vr was also closer to the line of the limiting parabola than the theoretical line of unity, and this supports previous analysis that additive effects are more important than dominance gene effects.

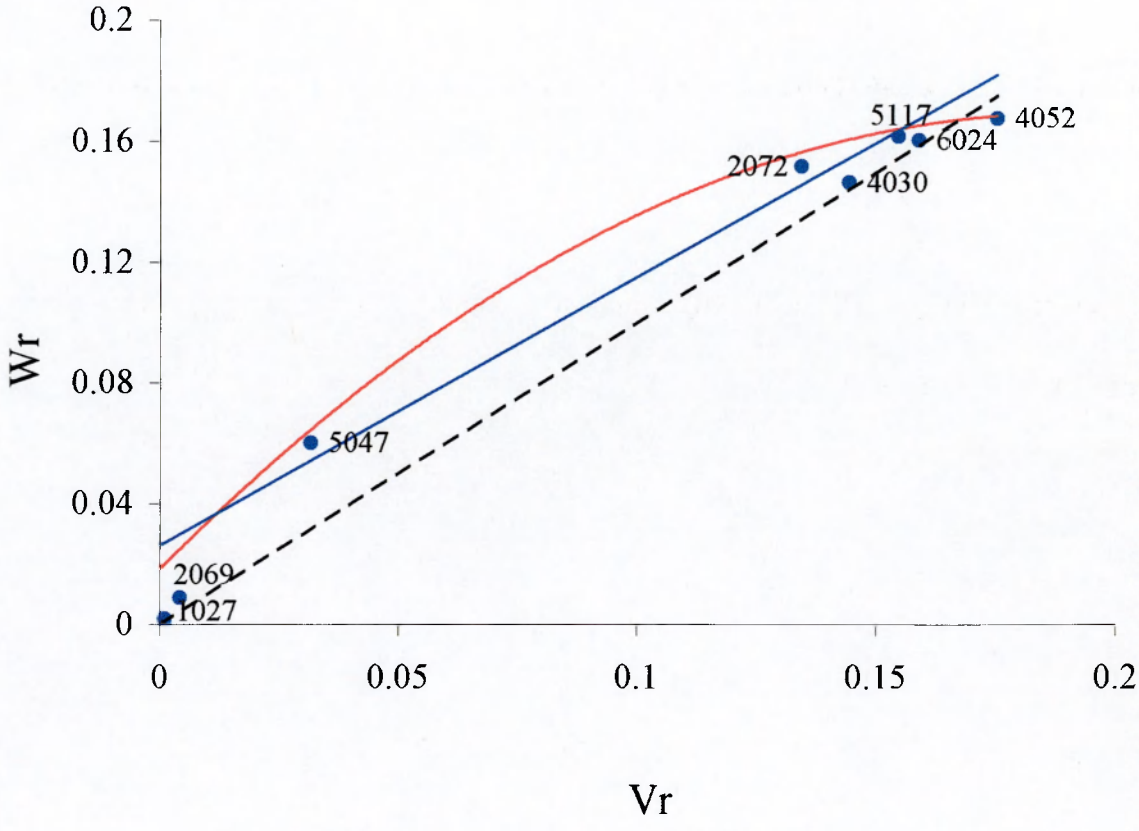
A plot of Wr + Vr against the mean parental value is shown in Figure 26. The lower the frequency of crown gall formation in the parental line the smaller the corresponding Wr + Vr, showing that the alleles for low gall formation (or resistance) are dominant to those for high gall formation. The plot of Wr + Vr against the mean parental value also gave a positive correlation coefficient, $r = 0.96$ ($P < 0.01$), indicating that recessive alleles act to increase expression of the character (*i.e.* recessive alleles correspond to high gall formation). Dominance also appears to be unidirectional, as indicated by an r-value close to 1.0.

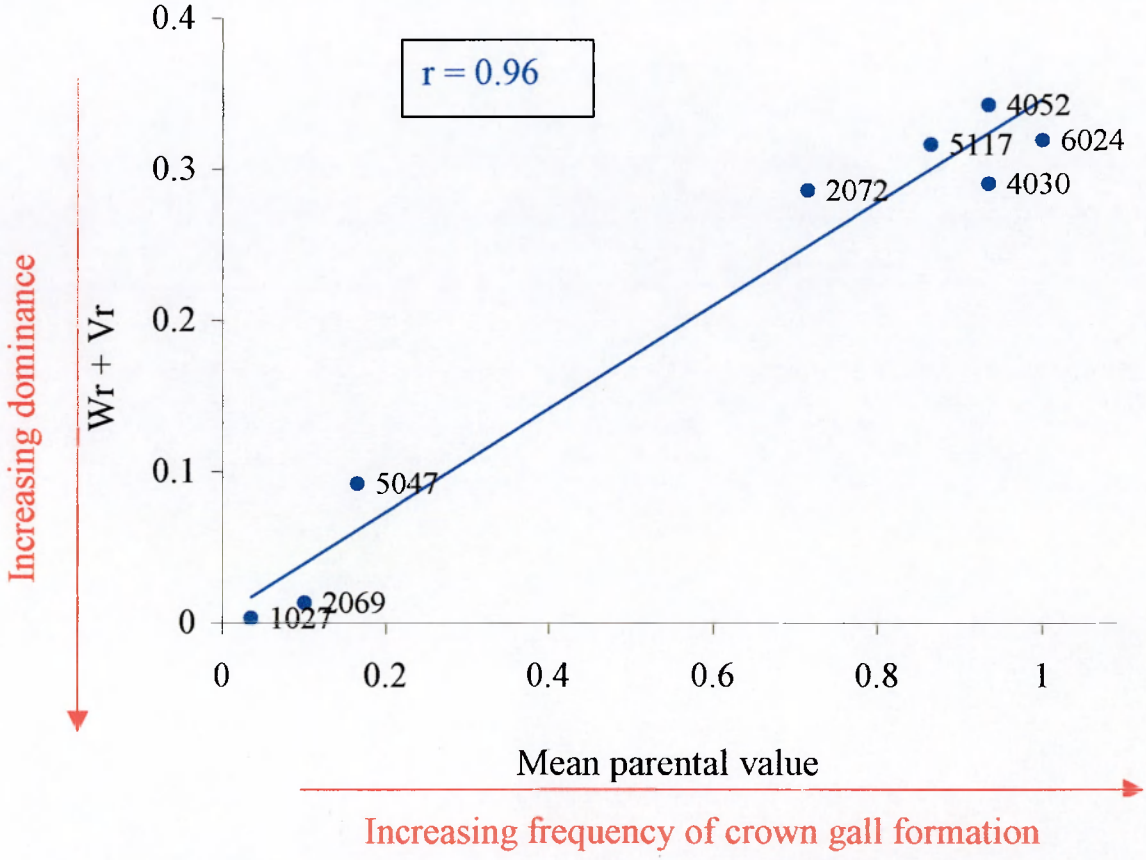
Figure 25 **The relationship between the variance of the F_1, for each parental line (Vr) and their covariance with the recurrent parent (Wr), for crown gall formation.**

• Plot of Vr vs Wr. The limiting parabola is shown in red, and the dashed line represents the theoretical line of unity.

Figure 26 **Wr + Vr from each array of the 8 x 8 diallel, plotted against the mean of the common parent.**

All points lie close to the line, indicating that the dominance relationship (for crown gall formation) holds true for all parents.


0.2
0.16
0.12
0.08
0.04
0
Wr
0
0.05
0.1
0.15
0.2
Vr
5117
4052
6024
2072
4030
5047
2069
1027


r = 0.96
0.4
0.3
0.2
0.1
0
Wr + Vr
0
0.2
0.4
0.6
0.8
1
4052
5117
6024
2072
4030
5047
2069
1027
Increasing dominance
Mean parental value
Increasing frequency of crown gall formation

The relationship between Vr and Wr, gave no reason to doubt the adequacy of the simple genetic model, as such the components of variation D, H_1, H_2, F and E were calculated to further investigate the genetic control of susceptibility towards *A. tumefaciens* (presented in Table 24).

Table 24 Genetic components analysis: Crown gall formation.

Component	
D	0.16
H_1	0.12
H_2	0.11
F	-0.099
E	0.009
Average degree of dominance $\sqrt{H_1/D}$	0.86
Proportion of dominance $H_2/4H_1$	0.22
Broad-sense heritability	0.95
Narrow-sense heritability	0.79

Additive variation (D) was larger than the dominance genetic variances (H_1 and H_2). This was noted also in Table 23 with MS values far greater for (a) additive effects than (b) dominant effects. The mean degree of dominance was 0.86, again indicating incomplete dominance. The broad sense heritability was 0.95 and this suggests that almost 95 % of the variation observed in crown gall formation was due to genetic effects (with just 5 % being due to environmental or non heritable effects). The narrow sense heritability was also high at 0.79, and suggests that almost 79 % of the genetic control will be a result of additive gene effects.

4.5.4 Conclusions from diallel

Analysis of the 8 x 8 diallel, to investigate the genetic control of susceptibility to *A. tumefaciens,* suggests that the variation observed within the F_1 hybrids was due to both additive and dominant gene effects. The analysis suggests additive gene effects to

be more important. Both the broad and narrow-sense heritability values were high. The broad sense heritability value suggested that 95 % of the observed variation was due to genetic effects. More importantly the narrow sense heritability provides a measure of the breeding value of a population, and measures the proportion of the variation that is due to the additive gene effects of genes in the population. The high narrow sense heritibility value of 0.79 shows that around 79 % of this trait is controlled by additive gene effects, and therefore the potential to introduce this trait into breeding material is obviously high.

4.6 The inheritance of susceptibility to *Agrobacterium tumefaciens*: Investigating backcrossed and F_2 populations

4.6.1 Introduction

Diallel analysis suggested susceptibility to *A. tumefaciens* to be controlled by a simple inheritance pattern. The model showed both additive and dominance gene effects to be significant, with additive gene effects accounting for approximately 79 % of the total variation. Such a high degree of additive gene effects suggest the introduction of this trait into subsequent generations is possible. In order to demonstrate the inheritance pattern of susceptibility to *A. tumefaciens*, a subset of the F_1 (from the 8 x 8 diallel) were backcrossed to both their DH parents reciprocally, and also self-pollinated to produce the F_2 population. These populations were then screened for susceptibility to *A. tumefaciens* (strain C58).

4.6.2 Method

Five families were selected for investigation. The families included a high x high, a low x low and 3 high x low crown gall crosses. The DH parents, backcrossed (BC) and F_2 populations of these families were screened for susceptibility to C58 as previously described.

As the number of BC seed available was a limiting factor this experiment was set up at the same time as the shoot regeneration trials. Cotyledons were removed for the regeneration trial from 4-day-old seedlings taking care not to damage the meristem and the following day the 5-day-old seedlings were inoculated with C58. The removal of the cotyledons did not affect subsequent growth as first leaves emerged soon after. Due to the size and complexity of this screen the experiment was established over four weeks. Approximately 25 seedlings were inoculated per DH parent line. For the BC populations approximately 100 seedlings were inoculated (made up of F_1 x Parent A, Parent A x F_1, F_1' (the reciprocal to F_1) x Parent A and Parent A x F_1') for Parent A and vice versa for Parent B, and approximately 200 seedlings inoculated for the F_2 populations. Due to the difficulty in generating F_1 seed, it was not possible to re-screen the F_1 at the same time as the DH, BC and F_2 populations. Controls were also established for each of the crosses. The presence or absence of crown galls was scored 50 days after inoculation.

4.6.3 Predicted frequency of crown gall formation

The information gained from screening the DH and F_1 lines of the 8 x 8 diallel were used to make predictions on the inheritance of susceptibility towards *A. tumefaciens* in the subsequent populations described here. The diallel screen suggested

that additive gene effects (79 %) predominantly controlled susceptibility towards *A. tumefaciens*. Dominance effects and environmental effects attributed to 16 % and 5 % of the total variation, respectfully. Low crown gall formation (or resistance to *A. tumefaciens*) appeared to be dominant over high crown gall formation, and the trend generally appeared to be unidirectional. Maternal effects also appeared to be non-significant, and suggests the genetic control to be nuclear rather than cytoplasmic (this enabled reciprocal data to be pooled in this section). Using this information the following predictions were made.

The DH parents of the five Families were screened at the same time as the BC and F_2 populations. The F_1 were not re-screened along side these populations, and therefore the first estimate made was that of the F_1 value. Assuming additive gene effects to be predominant the F_1 was estimated as the mid-point value between the two DH parents (*i.e.* (Parent A + Parent B)/2). As dominance effects also contribute to the inheritance of this trait it could be assumed that a frequency of crown gall formation below the estimated value might be obtained. Likewise, the frequency of crown gall formation in the BC populations was estimated as the mid-point of the estimated F_1 value and the appropriate DH parent (to which the F_1 had been backcrossed to). Even though more variation (segregation) is observed in a F_2 population, the mean of the population will be the same as the mean of the F_1 population from which it was derived. The estimated F_2 value was therefore the same value as the estimated F_1.

4.6.4 Results

Both the observed and the expected values, for each of the five Families screened are presented in Table 25. Crossing two genotypes together that both have a high frequency of crown gall formation (Family 1, DH 4052 x DH 2072) resulted in high crown gall

formation in subsequent generations. The observed crown gall formation was slightly higher than the expected mid-parent value for the BC and F_2 populations. This was also observed in the F_1 of this cross, shown in the 8 x 8 diallel (Table 22, page 124) with values higher than the two parents observed. This may be an example of heterosis (hybrid vigour) which is associated with increased heterozygosity (the condition of having one or more pairs of dissimilar alleles). In a cross between two low crown gall forming lines (Family 2, DH 2069 and DH 1027) the low phenotype is conserved in subsequent generations. However, again the observed values are slightly higher in the BC and F_2 generations than expected, and again this could be an example of heterosis. What should be considered is that in this screen 25, 100 and 200 explants were screened for the DH parent, BC and F_2 generations respectively. A higher number of explants were screened for the BC and F_2 populations as a larger degree of segregation was expected, screening just 25 explants in these two populations may have resulted in an inaccurate estimate of the actual value of the phenotype. Retrospectively, 25 explants of the DH parental lines may not have been an adequate number for consistent results. For example, in the original DH screen (Table 20), the 8 x 8 diallel (Table 22) and this current screen (Table 25), respectively, the DH parental values for crown gall formation have been in the range of:

DH 4052: 1.00, 0.94, 0.80
DH 2072: 0.84, 0.72, 0.40
DH 2069: 0.05, 0.10, 0.00
DH 1027: 0.16, 0.04, 0.04

Although the phenotype has been conserved; DH 4052 and 2072 remain high crown gall forming lines, and DH 2069 and 1027 are consistently low frequency crown gall forming lines, the actual values have fluctuated. Therefore, a margin of error can be applied to the predicted F_1 value, presented in

Table 25, which will also have an affect on the expected BC and F_2 values. With this in mind, the crosses between low and high crown gall forming lines (Families 3, 4 and 5) are considered.

In Family 3 (DH 4052 x DH 1027) the observed values were close to those of the expected values. However, the BC populations were slightly lower than the expected value. The diallel analysis suggested that in low x high crosses, low crown gall formation (or resistance to *A. tumefaciens*) was dominant. This may explain the lower than expected values in the BC populations.

In the low x high crosses of Families 4 and 5, backcrossing the F_1 to the low parent resulted in an observed crown gall frequency higher than expected. Whilst backcrossing the F_1 to the higher parent, and the F_2 generation both gave observed results similar to those expected.

It is hard to accurately predict whether any of the observed values are significantly different to those of the expected values. However, in each case the expected phenotype is always observed. The proportion of BC and F_2 generations that gave values higher than expected were greater than those that were lower than expected (values lower than the mid-parent value would indicate dominant gene effects). The data presented in Table 25 would suggest that, in agreement with the diallel analysis and genetic component analysis, additive gene effects play the major role in the control and inheritance of susceptibility towards *A. tumefaciens*.

Table 25 **Inheritance of susceptibility towards *Agrobacterium tumefaciens* in F_1, BC and F_2 populations.**

	Family 1 4052 (H) x 2072 (H)		Family 2 2069 (L) x 1027 (L)		Family 3 4052 (H) x 1027 (L)		Family 4 2069 (L) x 2072 (H)		Family 5 1027 (L) x 2072 (H)	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
Parent A	0.80	-	0.00	-	0.80	-	0.00	-	0.04	-
Parent B	0.40	-	0.04	-	0.04	-	0.40	-	0.40	-
F_1	-	0.60	-	0.02	-	0.42	-	0.20	-	0.22
BC to Parent A	0.75	0.70	0.10	0.01	0.55	0.61	0.15	0.10	0.36	0.13
BC to Parent B	0.66	0.50	0.16	0.03	0.14	0.23	0.23	0.30	0.36	0.31
F_2	0.76	0.60	0.19	0.02	0.43	0.42	0.45	0.20	0.43	0.22

4.7 Discussion

The formation of crown galls, following inoculation with wild-type *A. tumefaciens*, enabled a quick visual reference to test for genotype susceptibility towards *A. tumefaciens*. As a preliminary step, screening for the presence or absence of crown galls was an efficient score to use with such a large number of explants. It has been suggested that merely measuring the size and frequency of crown galls could be a misleading indicator of virulence of *A. tumefaciens* (Phuddephat *et al.*, 1996). Tumour induction and growth depend not only on the efficiency of gene transfer but also on the hormonal status of the plant. Ideally, the expression of introduced reporters, such as *gus* or the presence of opines, should be measured. However, due to the size of the screen and the time resources available, such precise screening was considered unpracticable at this stage of the investigation. The data presented in Chapter 5, demonstrate a strong correlation between crown gall formation and GUS expression ($r = 0.81$, $P < 0.01$), suggesting that the genetic control of 'crown gall formation', outlined in this chapter, does relate to susceptibility towards *A. tumefaciens* and not to the hormonal status of the plant (*i.e.* the forming of the gall itself).

Three wild-type strains of *A. tumefaciens* were used in this study, Ach5 (an octopine strain) and T37 and C58 (two noplaine strains). A number of disarmed *Agrobacterium* strains commonly used for *Brassica* transformation have been derived from these strains, e.g. LBA 4404 derived from Ach5 and EHA 101 from C58.

The controls used in this experiment provided information on two concerns: (1) Does treatment with Minimal A media alone cause any tissue culture blackening at the injection site. (2) To distinguish between sugary callus formation (a typical wound response) and crown gall formation. The majority of controls showed either bruising (a faint discoloration) at the injection site or a sugary callus formation to the injection site.

Blackening was observed at the injection site of a small proportion of the controls, but this blackening was not present in every explant of those genotypes (sugary callus noted in the rest). It may be that in those explants that blackened, the droplet of Minimal A had stayed in contact with the injection site longer than the others and therefore, as with cotyledonary petioles in the regeneration trials, this blackening could be attributed to contact with culture medium. However, although present, blackening was never extreme within a genotype. Explants injected with *A. tumefaciens* did not exhibit blackening at a level higher than that observed within the control explants, data not shown. A hypersensitive response (of extreme blackening) towards *A. tumefaciens* was therefore not observed within the populations screened. There was a clear distinction between sugary callus and crown gall formation. Variation in the speed of crown gall formation was apparent within the population, but the final 50 day score enabled the detection of the slower forming galls.

The distribution frequencies for crown gall formation across the DH population fell into classes of low and high crown gall formation, similar to that of the two parental lines. A proportion of the DH lines also showed an intermediate response to crown gall formation, so genotypes could be classified as showing either low, intermediate or high crown gall formation. This kind of distribution pattern (with 'defined' classes) would suggest that susceptibility to *A. tumefaciens* has a relatively simple inheritance pattern.

A putative QTL associated with susceptibility to *A. tumefaciens* was located on linkage group O9 of the genetic map of *B. oleracea*. The use of substitution lines confirmed the presence of this QTL and suggested that genes responsible for susceptibility were located within the central 38 % of linkage group O9, and could possibly be attributed to a 2.7 % region of this linkage group.

Recently Howell *et al.*, (2002) reported that all nine linkage groups of the *Brassica oleracea* genetic map have been assigned to each of the nine chromosomes of

the karyotype derived from mitotic metaphase spreads of the *B. oleracea* var. *alboglabra* line A12DHd using FISH (fluorescence *in situ* hybridisation). The majority of probes were BACs, (bacterial artificial chromosomes) with A12DHd DNA inserts, which gave clear reliable FISH signals. Integration of the cytogenetic and genetic linkage map was achieved with 22 probes representing 19 loci. Four chromosomes (2, 4, 7 and 9) are in the same orientation as their respective linkage groups (O4, O7, O8 and O6) whereas four chromosomes (1, 3, 5 and 8) and linkage groups (O3, O9, O2 and O1) are in the opposite orientation. The remaining chromosome (6) was believed to be in the opposite orientation. The cytogenetic map is an important resource for locating probes with unknown genetic map positions and is also being used to analyse the relationships between genetic and cytogenetic maps. In light of this work, it is suggested that genes associated with susceptibility to *A. tumefaciens,* identified on linkage group O9, may therefore be located on Chromosome 3 of the cytogenetic map of *B. oleracea.*

Analysis of the 8 x 8 diallel, to further investigate the genetic control of susceptibility towards *A. tumefaciens,* suggested the variation observed within the F_1 hybrids was due to both additive and dominant gene effects, with additive gene effects being more important. The broad sense heritability value suggested that 95 % of the observed variation was due to genetic effects, and therefore just 5 % could be attributed to non-genetic or environmental effects. The high narrow sense heritability value of 0.79 shows that around 79 % of this trait is controlled by additive gene effects, and therefore the potential to introduce this trait into breeding material is obviously high. This was demonstrated in section 4.6, with the BC and F_2 populations.

These studies have demonstrated that susceptibility to *A. tumefaciens* is a heritable trait, and the potential for introducing such genes into breeding programmes, to increase future transformation potential is high. Whether the introduction of

susceptibility (or conversely the removal of resistance genes) would be detrimental to breeding lines should also be addressed. Crown gall disease is not a commercial problem for *B. oleracea* (unlike woody / vine species) and GDDH33 (which showed a high frequency of crown gall formation) is derived from the commercial variety Green Duke. This would suggest that the presence of these genes is not detrimental to the variety.

In *Arabidopsis* (a model species and, like the *Brassica* genus, a member of the family Cruciferae) susceptibility was observed to be a heritable trait. Resistance to *Agrobacterium* in *Arabidopsis* has been associated with a deficiency in T-DNA integration (Nam *et al.*, 1997). Mysore *et al.*, (2000) showed that in *rat5* a histone H2A gene is disrupted. Complementation analysis and *RAT5* over expression indicated that H2A plays a role in *Agrobacterium* transformation. The T-DNA integration stage of transformation is blocked in the *rat5* mutant. This suggests that histone H2A (RAT5) plays an important role in illegitimate recombination of T-DNA into the plant genome. The histone H2A genes comprise of a small multigene family in *Arabidopsis*, the precise mechanism by which mutation of one of these genes can cause the *rat* phenotype, is as yet unknown. However, further work by this group (Mysore *et al.*, 2002) demonstrated that germ-line transformation (vacuum infiltration) was possible in these *rat* mutants. They proposed that root transformation (and perhaps transformation of all somatic tissues) requires 'factors' that are limiting in these tissues but that are abundant in germ-line tissues.

The *Arabidopsis* genome has been completely sequenced, and the *rat5*/T-DNA junction and wild-type RAT5 gene sequence have been published (GenBank Database, accession nos. AF204967 and AF204968 respectively). In Chapter 4, the RFLP probe pW233 was identified as being closely associated with susceptibility towards *A. tumefaciens* (section 4.4 page 120). A BLAST search of the *Arabidopsis* database with

the probe pW233 (courtesy of Martin Trick, John Innes Centre, UK) showed that pW233 had an association with chromosome 5 of *Arabidopsis.* Interestingly RAT5 was also located on chromosome 5, although the two sequences did not map to the same place[3]. It would be of future interest to see if the RAT5 sequence is associated with DH lines in the mapping population that showed high crown gall formation. The evolutionary relationship between *Brassica* and *Arabidopsis* means that genes or genetic loci associated with susceptibility, or resistance, to *A. tumefaciens* could potentially have been conserved.

The same A12DHd/GDDH33 population has been screened for adventitious and transgenic root production following inoculation with *Agrobacterium rhizogenes* (Cogan *et al.,* 2002). Three QTL were identified for adventitious root production on linkage groups O3, O5 and O7. Putative transgenic roots were screened for the presence of GFP after 35 days to enable the detection of stably expressing transgenes, and QTL associated with GFP root production were assigned to linkage groups O1, O3 and O7. The QTL identified on linkage groups O3 and O7 were common to both adventitious and transgenic root production. The screening of substitution lines in order to assign the precise function of these QTL was proposed. Cogan *et al.* (2002) also suggested that these QTL are likely to be important in T-DNA transfer and integration into the plant nuclear genome. The process of T-DNA transfer and integration is likely to be the same for both bacterial types (*A. tumefaciens* and *A. rhizogenes*), and it is reasonable to assume that QTL associated with T-DNA transfer and integration should also be the same for the two bacterial types.

These studies have successfully demonstrated that the genetic loci on linkage group O9 are associated with susceptibility to *A. tumefaciens.* Data presented in

[3] The absolute coordinates, pW233 17,695,129 and *rat5* 22,211,212 (in bases) of the matches were deduced using WU-BLASTIN 2.0MP [Gish, W. (1996-2002) http://blast.wustl.edu] against a database comprising the Arabidopsis pseudochromosome sequences, version c080501 downloaded from the MIPS FTP site (ftp://ftpmips.gsf.de/cress/).

Chapter 5 also demonstrate that those DH lines that contain the O9 region associated with high susceptibility to *A. tumefaciens,* also show higher levels of stable transformation events. Further work will enable the precise nature of this QTL to be determined, be it bacterial attachment, T-DNA transfer or T-DNA integration.

Chapter 5 The use of phenotypic markers for identifying successful candidates for transformation

5.1 Introduction

The ability to regenerate shoots, *in vitro,* is an important prerequisite for any transformation programme, but high regeneration ability alone is no guarantee of transformation success (as discussed in Appendix A). In Chapter 3 a range of regeneration abilities within a DH population was identified, and in Chapter 4 the same population was screened for susceptibility to *A. tumefaciens.* By comparing the results of these two screens, Table 6 and Table 20 respectively, it becomes apparent that there is no direct correlation between shoot regeneration ability and *A. tumefaciens* susceptibility ($r = 0.01$, $P > 0.05$). Selecting a genotype for transformation based only on regeneration potential, or selecting a genotype based solely on susceptibility to *A. tumefaciens,* will not be a good indicator of transformation potential.

In this chapter, phenotypic markers have been employed in an attempt to identify genotypes amenable to transformation. Genotypes were selected for transformation based on shoot regeneration potential and susceptibility towards *A. tumefaciens.* Whether a genotype regenerated shoots via a callus phase and / or was prone to tissue culture blackening was also taken into account when predicting transformation success. Genotypes were transformed with the *Agrobacterium* strain LBA 4404, and in some cases also with EHA 101. Both strains contained the construct p25Gi (previously described in Chapter 2 section 2.5.1). EHA 101 and LBA 4404 are both disarmed strains of *A. tumefaciens,* derived from the wild-type strains C58 and Ach5 respectively, and both are routinely used for *Brassica* transformation (Moloney *et al.,* 1989 and Irwin *et al.,* 1998).

5.2 Materials and Methods

5.2.1 Plant Material

The genotypes selected for initial transformations are listed in Table 26. The previously identified tissue culture phenotypes, associated with shoot regeneration and susceptibility towards *A. tumefaciens,* are indicated for each of the genotypes.

5.2.2 Experimental procedure

Genotypes were transformed as previously described within section 2.5, but modified so that 5mg/l kanamycin was used as the selection level for initial transformations (for reasons outlined in Appendix A, section A.5). Cotyledonary petioles were transferred to fresh selection medium after 23 days, and a proportion of explants (~ 100) were surrendered at this time to test for GUS expression (the protocol for the detection of GUS expression is detailed under section 2.6). PCR was carried out on kanamycin resistant (green) shoots to confirm the presence of the *nptII* and *gus* genes (detailed in section 2.7).

Table 26 Genotypes selected for transformation.

Predictions were made for transformation events[†] (measured as the number of explants with GUS expression at the cellular level / the number of explants screened). Genotypes that showed high susceptibility to Ach5 and showed no tissue culture blackening were predicted to have the highest proportion of transformation events. Genotypes showing intermediate susceptibility to Ach5 but high susceptibility to C58, and no tissue culture blackening, were predicted as having the next highest level of transformation events. Tissue culture blackening was predicted to lower the number of transformation events observed.

Prediction of transformation success[††]; Genotypes that showed high shoot regeneration and susceptibility to *Agrobacterium*, and no tissue culture blackening where predicted to be the best candidates for transforming. It was predicted that low shoot regeneration ability, low susceptibility to *Agrobacterium,* and / or the presence of tissue culture blackening would reduced the potential for transformation success.

Genotype	Shoot Regeneration Potential	Tissue culture blackening	Callus forming genotype	Susceptibility to Ach5	Susceptibility to C58	Prediction of transformation events[†]	Prediction of transformation success[††]
SL172	Intermediate	Yes	No	Not tested	High	✓✓	✓
SL177	Intermediate	Yes	No	Not tested	Low	x	x
SL141	Intermediate	Yes	No	Not tested	High	✓✓	✓
SL142	Intermediate	Yes	No	Not tested	Low	x	x
A12	Intermediate	Yes	No	Low	Low	x	x
1027	Low	No	swelling	Low	Low	✓	x
3235	Intermediate	Yes	No	High	High	✓✓	✓
4052	Intermediate/High	Yes	No	High	High	✓✓	✓
6024	Intermediate	No	swelling	Intermediate	High	✓✓(✓)	✓(✓)
5117	High	No	swelling	High	High	✓✓✓	✓✓(✓)
1012	High	No	Yes	Intermediate	High	✓✓(✓)	✓✓(✓)
5118	Low	Yes	No	Int'/High	High	✓✓	x
5047	High	No	Yes	Low	Low	✓	✓
F_1 P14 (5047x5118)	High	Yes	No	Not tested	Not tested	?	?

[†] Transformation events: Gus expression at the cellular level:

[††] Transformation success: Production of transgenic shoots: ✓ ✓✓ High ✓✓ Good ✓ Fair x Poor ? Uncertain

5.3 Results

5.3.1 Transformation of plant cells by LBA 4404 harbouring p25Gi

After 23 days in culture approximately 100 explants per genotype were tested for expression of the *gus* gene. Following histochemical analysis (described in section 2.6) blue coloration of the plant cells was taken as a positive sign of transformation events, where cells had taken up, incorporated and expressed GUS. The *gus* gene contained an intron, and therefore any GUS expression was a result of transfer to the plant, and not expression from within the *Agrobacterium*. Any GUS activity detected early after infection (within the first few days) most probably represents transient expression of T-DNA that is not yet integrated into the plant genome (Janssen and Gardner, 1989), screening after 23 days enabled the detection of stabily incorporated T-DNA. The results from this screen are presented in Table 27, along side the results from the previous wild-type screen (where appropriate).

LBA 4404 is a disarmed *A. tumefaciens* strain derived from the wild-type Ach5. It was predicted that genotypes that had high susceptibility to Ach5 would also have a high susceptibility to LBA 4404. The results in Table 27 confirmed this prediction as genotypes that were more susceptible to Ach5 also showed high susceptibility to LBA 4404. The substitution lines SL 141 and SL 177 both contain the GDDH33 alleles associated with high susceptibility to *Agrobacterium* (section 4.3). It was therefore assumed that these lines would transform at a rate higher than SL 142, SL 172 and A12 (genotypes containing alleles associated with low susceptibility) when infected with LBA 4404.

Table 27 Transformation frequencies.

Following infection with the disarmed *A. tumefaciens* strain LBA 4404, transformation events were measured as the number of explants testing positive for GUS expression / the number of explants screened. The results are compared with infection by two wild-type strains (Ach5 and C58) of *A. tumefaciens* (measured as the number of explants producing crown galls / the number of explants screened).

Genotype	Blackening	Ach5 Gall Formation	C58 Gall Formation	Prediction of transformation events	LBA 4404 Gus Expression
SL 142	Yes	*	0.08	x	0.06
SL 172	Yes	*	0.05	x	0.16
A12	Yes	0.00	0.08	x	0.20
DH 5047	No	0.15	0.15	✓	0.30
DH 1027	No	0.00	0.16	✓	0.33
SL 177	Yes	*	0.90	✓✓	0.48
DH 4052	Yes	0.70	1.00	✓✓	0.48
SL 141	Yes	*	0.90	✓✓	0.49
DH 5118	Yes	0.66	1.00	✓✓	0.49
DH 6024	No	0.41	0.96	✓✓(✓)	0.49
F_1 P14 (5118 x 5047)	Yes	*	*	?	0.52
DH 3235	Yes	0.92	0.96	✓✓	0.53
DH 1012	No	0.55	1.00	✓✓(✓)	0.76
DH 5117	No	0.69	0.88	✓✓✓	0.90

* Not screened with this strain.
Level of transformation events predicted, ✓ ✓✓ high, ✓✓ good, ✓ low, x poor, ? uncertain.

Transformation events with LBA 4404 were again higher in SL 141 and SL 177 (Table 27) transforming at a rate of 0.49 and 0.48, whereas SL 142, SL 172 and A12 transformed at rates of 0.06, 0.16 and 0.20 respectively. These results again support the significance of the QTL on linkage group O9, and demonstrate that by introducing this region, from GDDH33, the transformation potential of SL 141 and SL 177 can be significantly increased. GUS expression (as well as crown gall formation) was much higher in SL 141 and SL 177, and this supports the theory that the region identified on

O9, is in fact associated with susceptibility to *Agrobacterium*. Confirming that the QTL is truly associated with susceptibility towards *Agrobacterium*, and is not an artefact of the hormonal status of the plant *i.e.* simply the plant's ability to form galls.

Genotypes that showed relatively low levels of susceptibility towards crown gall formation (the first five genotypes in Table 27) were predicted to respond poorly to the disarmed strain, LBA 4404. These genotypes on the whole did respond less well than those genotypes that were highly susceptible to crown gall formation. The variation in GUS expression levels between the low susceptible genotypes was interesting. Genotypes that experienced tissue culture blackening tended to show lower levels of transformation events. This is perhaps not surprising as tissue culture blackening will result in necrosis of cells at the cut surface of the explant, the high proportion of necrotic cells will obviously mean less GUS expression. In contrast, genotypes that did not exhibit tissue culture blackening, but whose petiole bases swelled and produced callus, had a much higher level of transformed cells. The same was seen in genotypes that had a high level of susceptibility to the wild-type strains of *Agrobacterium*; those that blackened (black text in Table 27) generally had lower transformation rates than those that did not blacken (green text in Table 27).

Of course, genotypes that do not blacken, but instead experience swelling and callusing to the petiole base may also be more attractive to *Agrobacterium*. Actively dividing cells are reported as being better targets for *Agrobacterium* (Nadolska-Orczyk *et al.*, 2000). This may also explain why higher levels of transformation events (GUS expression) were noticed in these low susceptibility lines, when cotyledonary petioles were infected as opposed to the inoculation of seedling hypocotyls where callus initiation is minimal.

Susceptibility to wild-type *Agrobacterium* was a successful indicator of a genotype's transformation potential with LBA 4404, at the cellular level. Although LBA 4404 is derived from Ach5, there was a higher correlation between GUS expression in cotyledonary petioles inoculated with LBA 4404 and crown gall formation in hypocotyl explants inoculated with C58, $r = 0.81$, $P < 0.01$ (c.f. Ach5 and LBA 4404 where $r = 0.68$, $P < 0.01$). Ach 5 was the least virulent of the wild-type *Agrobacterium* strains used. The lower correlation between Ach5 and LBA 4404 may be a result of the small number of explants originally screened with Ach5. For example, A12 and DH 1027 both failed to produce crown galls when infected with Ach5, but when infected with the more virulent strain, C58, both produced crown galls. It is highly likely that both genotypes would have produced crown galls with Ach5 if a larger number of explants had been screened.

5.3.2 Generation of transgenic plants

Susceptibility to wild-type strains of *Agrobacterium* was a reasonable indicator of a genotype's ability to be transformed, at the cellular level, with a disarmed strain of *Agrobacterium.* Genotypes that did not exhibit tissue culture blackening had higher levels of transformation events, than genotypes that did show signs of blackening. Even genotypes with low susceptibility to wild-type *Agrobacterium* (as determined by gall formation) had reasonable levels of transformation, with rates of up to 0.33, in genotypes that formed callus, and therefore did not blacken.

The affect of tissue culture blackening on reducing shoot regeneration potential was discussed in Chapter 3. High shoot regenerating genotypes were observed that also exhibited tissue culture blackening, in these genotypes the regenerated shoots tended to be much smaller and few in number, in contrast to genotypes that did not blacken, which callused and produced multiple shoots. Under 'transformation conditions' shoot

regeneration rates are often reduced, due to selection pressures and the process of infection. It is reasonable to assume that as tissue culture blackening appeared to reduce not only shoot regeneration potential but also cell transformation efficiency, genotypes that blackened would have a lower rate of transformation success (production of transgenic shoots) than non blackening genotypes. These observations were used to predict a genotype's transformation success, see Table 26.

All genotypes that exhibited tissue culture blackening failed to regenerate transgenic shoots (despite individual cells being transformed). In fact, shoot regeneration of non-transgenic shoots was also negligible in genotypes that blackened, under transformation conditions. Blackening of the cut petiole base was shown to be a strongly heritable trait. Crossing DH 5118 (a line that blackens) with DH 5047 (a line that produces a mass of callus) resulted in blackening in the F_1 hybrid (P14) with no callus formation. This demonstrates that blackening is dominant to non-blackening. The level of transformation events (% explants expressing GUS) was higher in P14 than either of the two parental lines. This was also seen in some of the F_1 crosses (diallel Table 22) where crossing two genotypes with similar susceptibility rates e.g. DH 2072 (0.72) x DH 4030 (0.90) gave rates of crown gall formation in the F_1 hybrids of between 0.86-1.00 (*i.e.* sometimes above that of the two parents). The presence of blackening in P14 explants makes this genotype an unsuitable candidate for transformation, and despite cells being transformed and having shoot regeneration potential (shown in Table 8) no transformed shoots were regenerated. Clearly showing that tissue culture blackening is a strong limiting factor to transformation success.

Transgenic plants that were both *nptII* and *gus* PCR positive (PCR data not presented) were recovered from all genotypes that had a phenotype of intermediate to high shoot regeneration, and did not exhibit tissue culture blackening. Cotyledonary petioles of DH 6024 did not blacken and showed slight swelling at the petiole base. For

this genotype, shoot regeneration was at the intermediate level, as was its response to *Agrobacterium.* It was predicted that this genotype had potential as a candidate for transformation, but that its intermediate regeneration potential and susceptibility to *Agrobacterium* may be limiting factors for efficient transformation. Transgenic shoots were recovered at a rate of 0.4 % (see Table 28). DH 5117 also showed slight swelling of the petiole base and was not effected by blackening. This genotype had a high shoot regeneration response and high susceptibility to *Agrobacterium*, and as such was expected to respond better than DH 6024. However, transgenic plants were recovered from DH 5117 at a rate of 0.5 % (similar to that of DH 6024). In contrast, transgenic plants were recovered from DH 1012 at a rate of 9.6 %. This genotype differed from DH 6024 and DH 5117 because it regenerated multiple shoots in association with an obvious callus phase, rather than few shoots via swelling of the petiole base. Shoot regeneration from this genotype was not affected by the 'transformation environment' to the degree that other genotypes were, and a high regeneration of non-transformed shoots was still maintained in the presence of kanamycin. The level of selection (5 mg/l) was high enough to cause chlorosis of regenerating non-transformed shoots, however, increasing the level of selection to 15 mg/l slowed down shoot regeneration and resulted in the recovery of up to 31 % transgenic shoots, (in a subsequent transformation, see Table 28) when measured as the number of positive transgenics produced / the number of explants infected. Time restraints on this project meant it was not possible to confirm, by Southern analysis whether multiple transgenic shoots taken from the same explant were a result of independent transformation events. However, it is certain that the number of independent transgenics produced was at least 20 %, based on the number of explants producing transgenic shoots / the number of explants infected.

Not only was this rate higher than anything reported in the literature to date, for the transformation of cotyledonary petioles of *B. oleracea* (up to 7.7 % reported by Irwin *et al.*, 1998, based on the proportion of individual transgenic plants produced), but the speed of regeneration within this DH line also enabled transgenic shoots to be recovered just 4 weeks after inoculation. DH 1012 was subsequently transformed with the disarmed strain EHA 101 (derived from C58) a more virulent strain of *Agrobacterium* in order to see if transformation rates could be increased further. The data shown in Table 28 indicate that transformation rates were not significantly altered. It should be noted that this transformation was carried out at a separate time from the LBA 4404 transformation, and was based on observations from just one transformation. The results however, do show that transformation rates for DH 1012 are consistently high and repeatable, using different strains of *Agrobacterium.*

DH 5047 was also a high shoot regenerating genotype that did not blacken and produced multiple shoots via a prolific callus phase. This genotype had low susceptibility to the wild-type *Agrobacterium* strain Ach5. Based on shoot regeneration response it was assumed that this genotype would be a successful candidate for transformation with LBA4404, but that the efficiency of transformation would be limited by the genotypes low susceptibility to *Agrobacterium* infection. Transgenic plants were obtained from DH 5047 following transformation with LBA 4404 (Table 28) at a transformation rate of 4.5 %, confirming that it is the mode of shoot regeneration that is the major limiting factor for efficient transformation. If higher susceptibility to *Agrobacterium* could be introduced into this line, it is possible that transformation efficiencies would be increased further. DH 5047 was also transformed with EHA 101, and transgenic plants recovered. The rate of transformation was similar to that obtained when transformed with LBA 4404. Again showing DH 5047 can be repeatedly transformed at a consistent level.

Table 28 Production of transgenic plants.

Genotype	No. Explants [1]	% Transgenic plants produced [2]	% Individual transgenic plants produced [3]
1012	624	88/624 = 14 %	60/624 = 9.6 %
1012 [a]	480	150/480 = 31 %	97/480 = 20 %
1012 [b]	370	86/370 = 23 %	57/370 = 15 %
6024	250	1/250 = 0.4 %	1/250 = 0.4 %
5117	580	3/580 = 0.5 %	3/580 = 0.5 %
5047	400	20/400 = 5.0 %	18/400 = 4.5 %
5047 [b]	330	23/400 = 5.8 %	21/400 = 5.3 %

1) Number of explants remaining after 100 were surrendered for GUS testing.

2) Total number of green shoots isolated that tested positive for GUS / total number of explants. (Whether all the shoots produced from one explant came from independent transformation events or from a shared event is not known, so this value may include clones).

3) Total number of explants that produced *gus* and *nptII* positive shoots / total number of explants (a measure of independent transformation events).

a) A second transformation was carried out with 1012, this time using 15 mg /l kanamycin as the selection level for transformation, this level of selection was used for subsequent transformations, with this genotype.

b) Transformation with *Agrobacterium* strain EHA 101 (derived from C58).

5.4 Discussion

A low correlation between high shoot regeneration and susceptibility to *A. tumefaciens* has shown that the use of each character alone would not be a good indicator of transformation success. For example, a genotype may show high susceptibility to *A. tumefaciens* but have low shoot regeneration potential, which would act as a strong limiting factor for its transformation success.

The production of crown galls was a good indicator of positive transformation events. Genotypes that were susceptible to the wild-type *A. tumefaciens* were also

susceptible to transformation by disarmed strains of *A. tumefaciens*, as indicated by the expression of the *gus* gene by the plant genome. The *gus* gene contained an intron, so expression after 23 days was a result of uptake into the plant genome, and not a result of transient or bacterial expression. The substitution lines that contained the GDDH33 region of linkage group O9, associated with high crown gall formation, also had a higher level of transformation efficiency than those lines containing the A12DHd region of O9. This suggests that the region identified on O9 is associated with susceptibility to *A. tumefaciens*. The transfer of these genes, into otherwise recalcitrant lines (Table 27) also demonstrated the ability to increase transformation efficiency, at the cellular level.

Susceptibility to *A. tumefaciens* and shoot regeneration potential are both useful phenotypic markers to assist in selecting genotypes for transformation. However, the blackening / callus phenotype also appears to be a strong, if not critical, factor for transformation. Transgenic plants were not recovered from any genotypes that exhibited tissue culture blackening, despite some of these genotypes having high *Agrobacterium* susceptibility and shoot regeneration potential. Shoot regeneration potential was reduced under transformation conditions, especially in genotypes showing tissue culture blackening.

The negative effects of tissue culture blackening on the regeneration of transformed shoots have been reported for a number of species, e.g. grape (Colby *et al.*, 1991 and Perl *et al.*, 1996) and soybean (Olhoft *et al.*, 2001a/b), discussed further in section 3.5. In soybean (Olhoft *et al.*, 2001a) the addition of L-Cysteine to the culture medium reduced tissue culture browning and transformation infection rates rose from 37 % to 91%.

In this present study, tissue culture blackening was thought to be a result of a reaction between the explant (perhaps associated with chemicals released as part of the plant defence mechanism) and the culture medium (illustrated in Figure 4 page 51). It

would therefore be of further interest to see if the inclusion of antioxidants into the culture medium would have an effect on the extreme tissue culture blackening demonstrated by some of the genotypes used in this study. Elimination or reduction of blackening may then in turn have an affect on increasing the transformation efficiency of these genotypes.

Genotypes that did not blacken and regenerated multiple shoots via a callus phase showed the greatest level of transformation success, DH 1012 and DH 5047, even with differing levels of susceptibility towards *Agrobacterium*. The correct mode of shoot regeneration appeared to be the more critical factor. In DH 5047 the level of susceptibility (measured as GUS expression) was higher in the cotyledonary petiole base than at the hypocotyl injection site with wild-type *A. tumefaciens*. This could be due to a number of reasons. (1) Different tissue types have been reported to show differing levels of susceptibility to *A. tumefaciens* (Akama *et al.*, 1992), or (2) cotyledonary petioles of DH 5047 went through a callus phase and it is thought that these actively dividing cells are a more attractive target to *A. tumefaciens*. Indeed, a number of transformation protocols often start with a callus initiation stage first, and use callus tissue as a starting point for transformation (Nadolska-Orczyk *et al.*, 2000).

Genotypes that tested GUS positive (at the explant stage) did so to different degrees. Genotypes that did not go through a callus phase often had single blue dots, whereas genotypes that initiated callus had a larger area of GUS expression. Having a larger number of transformed cells may result in a higher proportion of transgenic shoots regenerated.

5.5 Conclusions

Tissue culture blackening appeared to be the strongest limiting factor for transformation; lines that were high shoot regenerating phenotypes and had high

susceptibility for *Agrobacterium* did not regenerate transgenic shoots if they experienced tissue culture blackening. If blackening could be eliminated it would be interesting to see if this would have an effect on the regeneration of transformed shoots, as seen in soybean (Oloft *et al.*, 2001a). Investigation into the elimination of blackening is an obvious first choice for increasing transformation efficiency, before the introduction of genes for high/multiple shoot regeneration, or high susceptibility towards *Agrobacterium,* are considered for use in recalcitrant genotypes.

Of the genotypes that were successfully transformed the two most efficient genotypes were DH 5047 and DH 1012. Both genotypes regenerate multiple shoots at a high frequency via a callus phase and do not exhibit tissue culture blackening. However the two genotypes differ in their response to *Agrobacterium*. DH 1012 has higher transformation efficiency, was more susceptible to *Agrobacterium*, and therefore will have more transformed cells from which transgenic shoots regenerate. Therefore, for genotypes that regenerate in the desired way it may be optimal to increase *Agrobacterium* susceptibility, in order to increase transformation efficiency.

DH 1012 consistently produced transgenic plants at a high rate. The results of the transformations with LBA 4404 and EHA 101 illustrate the efficiency of the transformation system in terms of the total number of plants produced, the number of independent transgenic lines produced and the speed of production of transgenic shoots and flowering plants. DH 1012 is also highly self-compatible enabling the efficient production of seed for subsequent generations. The rapid production of a high frequency of transgenic plants demonstrate the efficacy of this DH line, from a mapped reference population, for use as a model genotype for testing gene function in *Brassica*.

Chapter 6 General Discussion

This study set out to elucidate the genetic control of *in vitro* shoot regeneration and the production of transgenic plants. Results from this research have shown that shoot regeneration, susceptibility to *Agrobacterium tumefaciens* and transformation efficiencies are all governed by plant genotype. Using knowledge gained from the studies presented in Chapters 3 and 4, candidate genotypes were selected for transformation (Chapter 5). A high throughput genotype, DH 1012, was identified that not only produced transgenic shoots at a rate higher than anything previously reported, for the transformation of cotyledonary petioles (7.7 % Irwin *et al.*, 1998 and 3 % Tsukazaki *et al.*, 2002), but the speed and efficiency of regeneration enabled the removal of transgenic shoots just 4 weeks after inoculation (*c.f.* 3 months reported by Tsukazaki *et al.*, 2002). The 'optimal phenotype' for transformation appeared to be a genotype with high shoot regeneration potential, that produced multiple shoots per explant via a swelling or callus phase at the petiole base. Both high and multiple shoot regeneration were shown to be heritable traits, with additive gene effects accounting for the majority of the genetic control. The potential to increase shoot regeneration in subsequent generations was successfully demonstrated in section 3.4 when the regeneration potential of parent, F_1, F_2 and backcrossed generations were investigated.

The absence of tissue culture blackening was shown to be a critical factor for successful transformation, as transgenic shoots were only recovered from genotypes that did not exhibit tissue culture blackening. The number of transformation events in genotypes exhibiting tissue culture blackening was also lower than non-blackening genotypes. In Chapter 3 it was proposed that this tissue culture blackening might have an inhibitory effect on shoot regeneration potential. The use of antioxidants in other species to successfully reduce browning and increase transformation events has been

reported, for example, in soybean (Olhoft *et al.*, 2001a) the addition of L-Cysteine to the culture medium reduced tissue culture browning, resulting in a considerable rise in the observed number of transformation events.

Tissue culture blackening was shown to be the dominant phenotype over non-blackening phenotype. This was demonstrated (in Chapter 5) by the cross between DH 5047, a non-blackening genotype and DH 5117 a genotype that did exhibit blackening (Table 27, page 145), where tissue culture blackening was shown to be passed onto the resulting hybrid. In the two original parents of the DH population, A12DHd exhibited tissue culture blackening to a high degree, while GDDH33 was a non-blackening genotype. A high proportion of DH lines within the screened population also exhibited the dominant phenotype of tissue culture blackening. If, as proposed, tissue culture blackening reduces the shoot regeneration potential of cotyledonary petioles, its removal should correlate with an increase in shoot regeneration across the DH population. A putative QTL associated with shoot regeneration was identified on linkage group O1 of *B. oleracea*. The QTL, although statistically significant (with an LOD score above the threshold value of 3.0), was only marginally above the level of significance (Figure 6, Chapter 3) and as such was viewed with slight caution. If the removal of tissue culture blackening does have a positive effect on shoot regeneration, the significance of the QTL on O1 may increase, confirming the importance of this region in association with shoot regeneration from cotyledonary petioles.

The results of the diallel analysis showed that a simple 'additive-dominant' genetic model controlled shoot regeneration from cotyledonary petioles, with additive gene effects being more important. The strong presence of additive gene effects highlights the potential for this trait to be selected for in subsequent generations, by conventional plant breeding, to increase the shoot regeneration potential of breeding/ research material. These results are fully supported by the findings of Ono *et al.*,

(2000), where the genetic control of shoot regeneration from cotyledonary petioles of *B. napus* was also shown to be controlled by additive and dominant gene effects with additive effects again being more important. Diallel analysis in both studies demonstrated that crossing a genotype that contained positive alleles for shoot regeneration with an unresponsive genotype resulted in an intermediate response in the hybrid. Likewise, studies reported by Narasimhulu *et al.*, (1988 a,b) demonstrated that crossing *B. oleracea* genotypes with high shoot regeneration potential with the recalcitrant genotypes of *B. rapa* resulted in an intermediate shoot regeneration response in *B. napus,* the interspecific hybrid. The results from this present study suggest that genes responsible for shoot regeneration have been conserved within the *Brassica* genus. This is important if the desirable phenotypes, identified as being important for efficient transformation, which includes high shoot regeneration, are to be transferred across the species.

High susceptibility to *Agrobacterium tumefaciens* was shown to have a positive effect on transformation efficiency. A putative QTL associated with susceptibility to *A. tumefaciens* was located on linkage group O9 of the genetic map of *B. oleracea.* The use of substitution lines confirmed the presence of this QTL, and suggested that genes responsible for susceptibility to *A. tumefaciens* were located within the central 38 % of linkage group O9, and could possibly be attributed to a 2.7 % region of this linkage group (Chapter 4). Following the alignment of the chromosomes and linkage groups of *B. oleracea,* Howell *et al.*, (2002), it is suggested that genes associated with susceptibility to *A. tumefaciens,* identified on linkage group O9, may be located on Chromosome 3 of the cytogenetic map of *B. oleracea.* Screening of the substitution lines also demonstrated that genes associated with high susceptibility to *A. tumefaciens* could successfully be transferred to recalcitrant lines, by methods of conventional plant breeding.

Analysis of an 8 x 8 diallel, to further investigate the genetic control of susceptibility towards *A. tumefaciens* (section 4.5.3), suggested that the trait is controlled by both additive and dominant gene effects, with additive gene effects being more important. The high proportion of additive gene effects suggested the potential to introduce this trait into breeding or research material is obviously high. The ability to increase susceptibility towards *A. tumefaciens* in subsequent generations was successfully demonstrated in section 4.6, when the inheritance of crown gall formation in the parent, F_1, F_2 and backcrossed generations was investigated. In Chapter 5 it was demonstrated that genotypes containing the region of O9 associated with high crown gall formation, also had higher rates of transformation events. So O9 is again confirmed to be associated with susceptibility to *A. tumefaciens.* These studies have demonstrated that susceptibility to *A. tumefaciens* is a heritable trait, and the potential for introducing such genes into breeding programmes, to increase future transformation potential is high.

It would be an over simplification to conclude that only these characters are important for successful transformation, but the importance of these traits has been demonstrated. It is possible that a non-blackening genotype with high susceptibility to *Agrobacterium* and high shoot regeneration potential may not produce transgenic shoots. This might be the case if the cells that regenerate are not the same as those targeted for transformation. The use of a non-destructive marker such as GFP (Green Fluorescent Protein) would be of benefit for the further investigation of target and regenerating cells. GFP would also be of benefit for detecting early transient expression and its comparison with stable expression at a later date. This would give further insight into what stage the genes associated with susceptibility to *Agrobacterium* relate to. Transient expression would show that bacterial T-DNA had been imported into the

plant cell, the failure to produce stable expression of the gene some time after infection would indicate that the T-DNA had not been integrated into the plant genome.

It would be of interest to see if the characters/ phenotypes identified as being the best candidates for transformation are shared outside of this population. The screening of breeding material of *B. olereacea* in order to identify successful candidates for transformation would be of benefit in confirming these characters as significant for transformation. Once verified these phenotypes could become routinely used for identifying the better candidates for transformation, reducing the effort spent on futile attempts with recalcitrant genotypes. When breeding material of *B. oleracea* was screened for transformation potential (Appendix A, section A.3) the genotype with the highest transformation efficiency had a high shoot regeneration potential, produced multiple shoots via a swelling of the petiole base and showed no tissue culture blackening. This genotype had high susceptibility to C58 and a low susceptibility to Ach5 (data not shown). This genotype therefore fits the profile identified for the selection of successful transformation candidates, within the DH population. These observations support the idea that the desired phenotypes (of high regeneration of multiple shoots, from genotypes that do not blacken and have susceptibility to *Agrobacterium*) do apply to *B. oleracea* genotypes outside of the DH population. It would be of particular interest to determine if these phenotypes also applied for other *Brassica* species.

Conclusions and Applications

This research provides a better understanding of why some genotypes remain recalcitrant to transformation. Desirable characteristics associated with successful transformation have been identified and these include high shoot regeneration potential, the production of multiple shoots via a swelling or callus phase at the petiole base and

susceptibility to *A. tumefaciens.* Tissue culture blackening was shown to be a critical factor limiting the production of transgenic shoots. The application of these characteristics to screen potential transformation candidates should enable the efficient identification of lines that favour transformation and the production of transgenic plants, and will prevent costly and futile efforts with genotypes that are likely to remain recalcitrant to transformation. Each of the 'desirable' characteristics identified have been shown to be heritable traits and the potential to select for favourable tissue culture traits in subsequent generations, using conventional breeding methods has been successfully demonstrated in this study.

The future of the commercial application of GM technology will understandably be governed by public concerns towards safety to public health and the environment. However, it is important to recognise the role of genetic transformation as an effective technology for understanding how genes function. The results of such studies will filter back into conventional breeding technologies. By applying the information gained from this present study the potential to identify model genotypes for each of the main *Brassica* species could be realised.

A high throughput genotype, DH 1012, was identified that enabled the removal of transgenic shoots just 4 weeks after inoculation. High transformation rates were consistently observed with this genotype when transformed with two different *A. tumefaciens* strains (EHA 101 and LBA 4404) both containing the same T-DNA construct (Table 28, Chapter 5). DH 1012 has subsequently been successfully transformed with the *A. tumefaciens* strain AGL1, containing a co-transformation system to later facilitate the removal of antibiotic reporter genes. It has also been transformed with LBA 4404 containing a different T-DNA construct (data not presented). The efficacy of DH 1012 as a potential model genotype for transformation has been demonstrated. The high efficiency of this genotype, as well as the association

with the genetic linkage map, will make this genotype very desirable as a potential research and development tool for studying gene function.

REFERENCES

Abeyasekera, S and R. Stern. 2001. Analysis of variances and its extended uses. Published by the Reading Statistical Services Centre, Reading University, UK.

Akama, K., H. Shiraishi, S. Ohta, K. Nakamura, K. Okada and Y. Shirmua. 1992. Efficient transformation of *Arabidopsis thaliana*: Comparison of the efficiencies with various organs, plant ecotypes, and *Agrobacterium* strains. Plant Cell Reports 12:7-11.

Babic, V., R. S. Datla, G. J. Scoles, and W. A. Keller. 1998. Development of an efficient *Agrobacterium*-mediated transformation system for *Brassica carinata*. Plant Cell Reports 17:183-188.

Bailey, M. A., H. R. Boerma, and W. A. Parrott. 1994. Inheritance of *Agrobacterium tumefaciens*-induced tumorigenesis in soybean. Crop Science 34:514-519.

Barfield, D. G. and E. C. Pua. 1991. Gene-transfer in plants of *Brassica juncea* using *Agrobacterium tumefaciens* mediated transformation. Plant Cell Reports 10:308-314.

Ben Amer, I. M., A. J. Worland and A. Borner. 1995. Chromosomal location of genes affecting tissue culture response in wheat. Plant breeding 114:84-85.

Ben Amer, I. M., V. Korzun, A. J. Worland and A. Borner. 1997. Genetic mapping of QTL controlling tissue-culture response on chromosome 2B of wheat (*Triticum aestivum*) in relation to major genes and RFLP markers. Theoretical and Applied Genetics 94:1047-1052.

Birhman, R. K., G. Laublin, and M. Cappadocia. 1994. Genetic control of *in vitro* shoot regeneration from leaf explants in *Solanum chacoense* Bitt. Theoretical and Applied Genetics 88:535-540.

Bliss, F. A., A. A. Almehdi, A. M. Dandekar, P. L. Schuerman, and N. Bellaloui. 1999. Crown gall resistance in accessions of 20 Prunus species. Hortscience 34:326-330.

Bouhuon, E. J. R., D. J. Keith, I. A. P. Parkin, A. G. Sharpe and D. J. Lydiate. 1996. Alignment of the conserved C genomes of *Brassica oleracea* and *Brassica napus*. Theoretical and Applied Genetics 93:833-839.

Bojsen, K., I. Donaldson, A. Haldrup, M. Joersboe, J. D. Kreiberg, J. Nielsen, F. T. Okkels, and S. G. Petersen. 1994: Mannose and xylose based positive selection. PCT-Int. Patent Application WO94/20627.

Buiatti, M., S. Baroncelli, A. Bennici, M. Pagliai, and R. Tesi. 1974. Genetics of growth and differentiation *in vitro* of *Brassica oleracea* var. *botrytis*. II An *in vitro* and *in vivo* analysis of diallel cross. Z Pflanzenzucht 72:269-274.

Carafa, A.M and G. Carratu. 1997. Stigma treatment with saline solutions: A new method to overcome self-incompatibility in *Brassica oleracea*. Journal of Horticultural Science 72:531-535.

Chen, J. L, and W. D. Beversdorf. 1994. A combined use of microprojectile bombardment and DNA imbibition enhances transformation frequency of canola (*Brassica napus*). Theoretical and Applied Genetics 88:187-192.

Christey, M. C. and E. D. Earle. 1991. Regeneration of *Brassica oleracea* from peduncle explants. Hortscience 26:1069-1072.

Churchill, G.A. and R.W. Doerge. 1994. Empirical threshold values for quantitative trait mapping. Genetics 138:963-971.

Colby, S.M., A. M. Juncosa, J. A. Stamp and C. P. Meredith. 1991. Developmental anatomy of direct shoot organogenesis from leaf petioles of *Vitis vinifera* (Vitaceae). American Journal of Botany 78:260-269.

Cogan, N. O. L., J. R. Lynn, G. J. King, M. J. Kearsy, H. J. Newbury and I. J. Puddephat. 2002. Identification of genetic factors controlling the efficiency of *Agrobacterium rhizogenes*-mediated transformation in *Brassica oleracea* by QTL analysis. Theoretical and Applied Genetics 105:568-576.

Coughtrey, A. and K. Mather. 1970. Interaction and gene association and dispersion in diallel cross where gene frequencies are unequal. Heredity 25:79-88.

David, C. and J. Tempe. 1988. Genetic transformation of cauliflower (*Brassica oleracea* var *botrytis*) by *Agrobacterium rhizogenes*. Plant Cell Reports 7:88-91.

De Block M., P. Tenning, and D. De Brouwer. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiology 91:694-701.

Frankenberger, E. A., P. M. Hasegawa, and E. C. Tigchelaar. 1981. Diallel analysis of shoot forming capacity among selected tomato genotypes. Zeitschrift Fur Pflanzenphysiologie 102:233-241.

Fry, J., A. Barnason, and R. B. Horsch. 1987. Transformation of *Brassica napus* with *Agrobacterium tumefaciens* based vector. Plant Cell Reports 6:321-325.

Gamborg, O.L., R. B. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research. 50:151-158.

Guerche, P., L. Jouanin, D. Tepfer, and G. Pelletier. 1987 a. Genetic transformation of oilseed rape (*Brassica napus*) by the Ri T-DNA of *Agrobacterium rhizogenes* and analysis of inheritance of the transformed phenotype. Molecular & General Genetics 206:382-386.

Guerche, P., M. Charbonnier, L. Jouanin, C. Tourneur, J. Paszkowski, and G. Pelletier. 1987 b. Direct gene-transfer by electroporation in *Brassica napus*. Plant Science 52:111-116.

Gupta, V., G. L. Sita, M. S. Shaila, and V. Jagannathan. 1993. Genetic transformation of *Brassica nigra* by *Agrobacterium* based vector and direct plasmid uptake. Plant Cell Reports 12:418-421.

Hansen, L. N. and E. D. Earle. 1994. Regeneration of plants from protoplasts of rapid-cycling *Brassica oleracea*. Plant Cell Reports 13:335-339.

Hansen, L. N., R. Ortiz, and S. B. Andersen. 1999. Genetic analysis of protoplast regeneration ability in *Brassica oleracea*. Plant Cell Tissue and Organ Culture 58:127-132.

Hayman, B. I. 1954. The analysis of variance of diallel crosses. Genetics 39: 789-809.

Hayman, B. I. 1957. Interaction, heterosis and diallel crosses. Genetics 42:336-355.

Hernadez-Fernandez, M. M, and B. R. Christie. 1989. Inheritance of somatic embryogenesis in alfalfa (*Medicago sativa.*). Genome 32:318-321.

Higgins, P., and R. J. Mathias. 1987. The effect of the 4B chromosomes of hexaploid wheat on the growth and regeneration of callus cultures. Theoretical and Applied Genetics 74:439-444.

Hoekema, A., P. Hirsch, P. Hooykaas, and R. Schilperoort. 1983. A binary plant vector strategy based on separate *vir* and T region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179-180.

Hood, E. E., G. L. Helmer, R. T. Fraley and M. D. Chilton. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in aregion of pTiBo542 outside of T-DNA. Journal of Bacteriology. 168:1291-1301.

Howell, E. C., G. C. Barker, G. H. Jones, M. J. Kearsey, G. J. King, E. P. Kop, C. D. Ryder, G. R. Teakle, J. G. Vicente and S. J. Armstrong. 2002. Integration of the cytogenetic and genetic linkage maps of *Brassica oleracea*. Genetics 161:1225-1234.

Irwin J. A., P. A. C. Sparrow and P. J. Dale. 1998. Transgene insertion and expression in horticultural brassicas. GCIRC Bulletin 13:32.

Jana, S. 1975. Genetic analysis by means of diallel graph. Heredity 35:1-19.

Janssen, B. J., and R. C. Gardner. 1989. Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Molecular Biology 14:61-72.

Jefferson, R.A., T. A. Kavanagh, and M. W. Bevan. 1987. Gus fusion: β-glucuronidase as a sensitive and versatile gene marker in higher plants. EMBO Journal 6: 3901-3907.

Jourdan, P. S., and E. D. Earle. 1989. Genotypic variability in the frequency of plant-regeneration from leaf protoplasts of 4 brassica spp and of *Raphanus sativus*. Journal of the American Society for Horticultural Science 114:343-349.

Kennedy, B. W and S. M. Alcorn. 1980. Estimates of U.S. crop losses to prokaryote plant pathogens. Plant Disease 64:674-676.

Kielly, G. A., and S. R. Bowley. 1997. Quantitative genetic analysis of *in vitro* callus proliferation in alfalfa. Canadian Journal of Plant Science 77:225-229.

Komatsuda, T., S. Enomoto, and K. Nakajima. 1989. Genetics of callus proliferation and shoot differentiation in barley. Journal of Heredity 80:345-350.

Komatsuda, T., T. Annaka, and S. Oka. 1993. Genetic mapping of a quantitative trait locus (QTL) that enhances the shoot differentiation rate in *Hordeum vulgare*. Theoretical and Applied Genetics 86:713-720.

Koornneef, M., C. J. Hanhart, and L. Martinelli. 1987. A genetic analysis of cell culture traits in Tomato. Theoretical and Applied Genetics 74:633-641.

Lazzeri, P. A. and J. M. Dunwell. 1984. *In vitro* shoot regeneration from seedling root segments of *Brassica oleracea* and *Brassica napus* cultivars. Annals of Botany 54:341-350.

Lazzeri, P. A. and J. M. Dunwell. 1986. *In vitro* regeneration from seedling organs of *Brassica oleracea* var *italica plenck* cv Green Comet .1. Effect of plant-growth regulators. Annals of Botany 58:689-697.

Lowe, J. M., M. R. Davey, J. B. Power and K. Blundy. 1993. A study of some factors affecting *Agrobacterium* transformation and plant regeneration of *Dendranthema grandiflora* tzvelev (syn. *chrysanthemum morifolium ramat.*). Plant Cell Tissue and Organ Culture 33:171-180.

Manninen, O. M. 2000. Associations between anther culture response and molecular markers on chromosomes 2H, 3H and 4H of barley (*Hordeum vulgare*). Theoretical and Applied Genetics 100:57-62.

Mano, Y., H. Takahashi, K. Sato, and K. Takeda. 1996. Mapping genes for callus growth and shoot regeneration in Barley (*Hordeum vulgare.*). Breeding Science 46:137-142.

Mano, Y. and T. Komatsuda. 2002. Identification of QTLs controlling tissue-culture traits in barley (*Hordeum vulgare* L.). Theoretical and Applied Genetics 105:708-715.

Mather, K. 1967. Complementary and duplicate gene interactions in biometric genetics. Heredity 22:97-103.

Mather, K. and J. L. Jinks. 1987. Biometrical Genetics. Third Edition. Chapman and Hall, London. Chapter 9:255-292.

Mauro, A. O., T. W. Pfeiffer, and G. B. Collins. 1995. Inheritance of soybean susceptibility to *Agrobacterium tumefaciens* and its relationship to transformation. Crop Science 35:1152-1156.

Molina, R. V. and F. Nuez. 1996. The inheritance of organogenic response in melon. Plant Cell Tissue and Organ Culture 46:251-256.

Moloney, M. M., J. M. Walker, and K. K. Sharma. 1989. High-efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Reports 8:238-242.

Mukhopadhyay, A., R. Topfer, A. K. Pradhan, Y. S. Sodhi, H. H. Steinbiss, J. Schell, and D. Pental. 1991. Efficient regeneration of *Brassica oleracea* hypocotyl protoplasts and high frequency genetic transformation by direct DNA uptake. Plant Cell Reports 10:375-379.

Murata, M. and T. J. Orton. 1987. Callus initiation and regeneration capacities in brassica species. Plant Cell Tissue and Organ Culture 11:111-123.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays and tobacco tissue culture. Physiol Plant 15:437-497.

Mysore, K. S., J. Nam, and S. B. Gelvin. 2000. An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration. Proceedings of The National Academy of Sciences of the United States of America 97:948-953.

Mysore, K. S., R. C. T. Kumar, and S. B. Gelvin. 2000 b. *Arabidopsis* ecotypes and mutants that are recalcitrant to Agrobacterium root transformation are susceptilble to germ-line transformation. The Plant Journal 21:9-16.

Nadolska-Orczyk, A., and S. Malepszy. 1989. *In vitro* culture of *Cucumis sativus* L. Genes controlling plant regeneration. Theoretical and Applied Genetic 78:836-840.

Nadolska-Orczyk, Orczyk and Przetakiewicz. 2000. *Agrobacterium*-mediated transformation of cereals - from technique development to its application. Acta Physiologia Plantarum 22:77-88.

Nam, J., A. G. Matthysse, and S. B. Gelvin. 1997. Differences in susceptibility of Arabidopsis ecotypes to crown gall disease may result from a deficiency in T-DNA integration. Plant Cell 9:317-333.

Nam, J., K. S. Mysore, C. Zheng, M. K. Knue, A. G. Matthysse, and S. B. Gelvin. 1999. Identification of T-DNA tagged Arabidopsis mutants that are resistant to transformation by *Agrobacterium*. Molecular and General Genetics 261:429-438.

Narasimhulu, S. B. and V. L. Chopra. 1988 a. Species-specific shoot regeneration response of cotyledonary explants of brassicas. Plant Cell Reports 7:104-106.

Narasimhulu, S. B., S. Prakash, and V. L. Chopra. 1988 b. Comparative shoot regeneration responses of diploid brassicas and their synthetic amphidiploid products. Plant Cell Reports 7:525-527.

Olhoft, P. M and D. A. Somers. 2001 a. L-Cysteine increases *Agrobacterium*-mediated T-DNA delivery in soybean cotyledonary-node cells. Plant Cell Reports 20:706-711.

Olhoft, P. M., K. Lin, J. Galbraith, N. C .Nielson and D. A. Somers. 2001 b. The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary-node cells. Plant Cell Reports 20:731-737.

Ooms, G., P. J. J. Hooykaas and G. Moolenaar. 1981. Crown gall plant tumors of abnormal morphology, induced by *Agrobacterium tumefaciens* carrying mutated octopine Ti plasmid – Analysis of T-DNA functions. Gene 14:33-50.

Ono, Y. and Y. Takahata. 2000. Genetic analysis of shoot regeneration from cotyledonary explants in *Brassica napus*. Theoretical and Applied Genetics 100:895-898.

Peng, J and T. K. Hodes. 1989. Genetic analysis of plant regeneration in rice (*Oryza sativa*). *In vitro* Cellular & Developmental Biology 25:91-94.

Perl, A., O. Lotan, M. Abu-Abied and D. Holland. 1996. Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.): The role of antioxidants during grape-*Agrobacterium* interactions. Nature Biotechnology 14: 624-628.

Poulsen, G. B. 1996. Genetic transformation of *Brassica*. Plant Breeding 115:209-225.

Puddephat, I. J., T. J. Riggs, and T. M. Fenning. 1996. Transformation of *Brassica oleracea*: A critical review. Molecular Breeding 2:185-210.

Puddephat, I. J., N. Thompson, H. T. Robinson, P. Sandhu, and J. Henderson. 1999. Biolistic transformation of broccoli (*Brassica oleracea* var. *italica*) for transient expression of the beta-glucuronidase gene. Journal of Horticultural Science & Biotechnology 74:714-720.

Quimin, C., and A and F. J. Zapata. 1990. Diallel analysis of callus induction and green-plant regeneration in rice anther culture. Crop Science 30:188-192.

Radke, S. E., J. C. Turner, and D. Facciotti. 1992. Transformation and regeneration of *Brassica rapa* using *Agrobacterium tumefaciens*. Plant Cell Reports 11:499-505.

Ramsey, L. D, D. E. Jennings, E. J. R. Bohuon, A. E. Arthur, D. J. Lydiate, M. J. Kearsey and D. F. Marshall. 1996. The construction of a substitution library of recombinant backcross lines in *Brassica oleracea* for the precision mapping of quantitative trait loci. Genome 39:558-567.

Ray, J.D., L. Yu, S. R. McCrouch, M. C. Champoux, G. Wang, and H. T. Nguyen. 1996. Mapping quantitative trait loci associates with root penetration ability in rice (*Oryza sativa*). Theoretical and Applied Genetics 92:627-636.

Sciaky, D., A. L. Montoya and M. D. Chilton. 1978. Fingerprints of *Agrobacterium* Ti plasmids. Plasmid 1:238-253.

Sebastian, R. L., E. C. Howell, G. J. King, D. F. Marshall, and M. J. Keasey. 2000. An integrated AFLP and RFLP *Brassica oleracea* linkage map from two morphologically distinct doubled-haploid mapping populations. Theoretical and Applied Genetics 100:75-81.

Sharpe A. G., I. A. P. parkin, D. J. Keith, and D. J. Lydiate. 1995. Frequent nonreciprocal translocations in the amphidiploid genome of oilseed rape (*Brassica napus*). Genome 38:1112-1121.

Szegedi, E. and P. Kozma. 1984. Studies in the inheritance of resistance to crown gall disease of grapevine. Vitis. 23:121-126.

Smith, R. H. and E. E. Hood. 1995. *Agrobacterium tumefaciens* transformation of monocotyledons. Crop Science 35:301-309.

Stam, P., and J. W. Van Ooijen. 1995. JoinMap™ version 2.0: Software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen.

Stover. E. W., H. J. Swartz and T. J. Burr. 1997. Crown gall formation in a diverse collection of *Vitis* genotypes inoculated with *Agrobacterium vitis.* American Journal of Enology and Viticulture. 48:26-32.

Taguchi-Shiobara, F., T. Komatsuda, and S. Oka. 1997 a. Comparison of two indices for evaluating regeneration ability in rice (*Oryza sativa*) through a diallel analysis. Theoretical and Applied Genetics 94:378-382.

Taguchi-Shiobara, F., S. Y. Lin, K. Tanno, T. Komatsuda, K. Yano, T. Sasaki and S. Oka. 1997 b. Mapping quantitative trait loci associated with regeneration ability of seed callus in rice, *Oryza sativa* L. Theoretical and Applied Genetics 95:828-833.

Takeuchi, Y., T. Abe, and T. Sasahara. 1997. Genetic analysis of plant regeneration from seed-derived calli in rice (*Oryza sativa*). Crop Science 37:963-965.

Tar'an, B., and S. R. Bowley. 1997. Inheritance of somatic embryogenesis in orchardgrass. Crop Science 37:1497-1502.

Taylor, T. E. and R. E. Veilleux. 1992. Inheritance of competences for leaf disk regeneration, anther culture, and protoplast culture in *Solanum-phureja* and correlations among them. Plant Cell Tissue and Organ Culture 31:95-103.

Tsukahara, M., T. Hirosawa, E. Nagai, H. Kato, R. Ikeda, and K. Maruyama. 1995. Genetic analysis of plant regeneration ability in cell suspension cultures of rice (*Oryza sativa*). Breeding Science 45:425-428.

Tsukazaki H., Y. Kuginuki, Y. Aida, and T. Suzuki. 2002. Agrobacterium-mediated transformation of a doubled haploid line of cabbage. Plant Cell Reports 21 (3): 257-262.

U. 1935. *Brassica napus.* Genomic analysis in *Brassica* with special reference to the experimental formation of and peculiar mode of fertilisation. Japan Journal of Botany. 7:389-452.

Vamos-Vigyazo, L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. CRC Critical reviews in food science and nutrition. 15 (1): 49-127.

Van Ooijen, J.W. and C. Maliepaard. 1996. MapQTL™ version 3.0: Software for the calculation of QTL positions on genetic maps. CPRO-DLO, Wageningen.

Van Ooijen, J.W. 1999. LOD significance thresholds for QTL analysis in experimental populations of diploid species. Heredity 83:613-624.

Van Sint Jan, V., G. Laublin, R. K. Birhman and M. Cappadocia. 1996. Genetic analysis of leaf explant regenerability in *Solanum chacoense*. Plant Cell Tissue and Organ Culture 47:9-13.

Wojtaszek, P. 1997. Oxidative burst: An early plant response to pathogen infection. Journal of Biochemisty 322:681-692.

Zambryski, P. 1988. Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annual Review of Genetics 22:1-30.

Zhang, L. and K. Hattori. 1998. Inheritance of high shoot regeneration ability from seed callus in a rice cultivar Joshu. Breeding Science 48:41-44.

Zhang, F. L. and Y. Takahata. 2001. Inheritance of microspore embryogenic ability in brassica crops. Theoretical and Applied Genetics 103:254-258.

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Appendix A

Identifying transformation variables in horticultural brassicas

A.1 introduction

A high shoot regeneration potential at the onset of a transformation programme is essential, as the infection and selection pressures incurred during the transformation process will greatly reduce the number of cells regenerating whole plants. It is also critical that genotypes selected for transformation are susceptible to *Agrobacterium* and that the cells targeted for infection are capable of regenerating shoots / whole plants. Finally it is important that selection is at a high enough level to give an advantage to transformed cells over their non-transformed counterparts. This Appendix reports the results from a series of preliminary studies carried out to identify variables associated with transformation efficiencies in horticultural *Brassica* species.

A.2 *In vitro* shoot regeneration in *Brassica*

One hundred and twenty four genotypes from three horticultural Brassica species (*B. rapa, B. oleracea* and *B. juncea*) were used to investigate *in vitro* shoot regeneration. The method used was a modified version of that described for the transformation of *B. napus* (Moloney *et al.*, 1989) and is detailed in section 2.3.

A.2.1 Plant material

B. rapa, B. juncea and *B. oleracea* were screened for *in vitro* shoot regeneration potential. Within each of the three species, genotypes were selected to cover a range of subspecies encompassing a number of seasonal varieties with a broad source of

geographical origins. Table A, indicates the number of genotypes screened for each of the *Brassica* species and acknowledges the source of the germplasm used.

Of the three horticultural brassicas selected, a heavier emphasis was placed on *B. oleracea*, and in particular Brussels sprout (*B. oleracea* ssp *gemifera*) and cabbage (*B. oleracea* ssp. *capitata*). The research on *B. oleracea* was undertaken as part of a MAFF funded project (HH1103SFV) to develop an efficient transformation system for horticultural brassicas.

A.2.2 Method

The method used for *in vitro* shoot regeneration, from cotyledonary explants is detailed in sections 2.3 and 2.5. For *B. rapa* the regeneration media was modified to contained 3 mg/l BAP and 1.5 mg/l NAA (following a screen of BAP and NAA combinations in order to determine optimal conditions for *B. rapa*). Explants were scored after 23 and 44 days in culture, for the presence or absence of shoots.

Table A Plant material

Brassica species	Subspecies or common name	Number of Genotypes	Acknowledgements for seed supply
B. rapa	*pekinensis*	3	HRI Wellesbourne [1] Svalof-Weibull[2]
	chinensis	5	
	parachinensis	1	
	oleifera	3	
	broccoletto	4	
	sylvestris	1	
	other (misc.)	9	
B. juncea	mustard	6	J.I.C Norwich [3]. HRI Wellesbourne[1] Svalof-Weibull[2]
	Chinese mustard	6	
	brown mustard	4	
	Japanese greens	2	
	large rooted - mustard	2	
	other (misc.)	8	
B. oleracea	kale	7	Elsoms Seeds[4] A.L. Tozers Ltd.[5]
	cauliflower	13	
	cabbage	19	
	Brussels sprout	19	
	Kohl rabi	4	
	broccoli	8	

Germplasm was supplied from

[1] Dr. D. Astley at the Genetic Resource Unit, Horticultural Research International, Wellesbourne, UK.

[2] Dr. K.Vamling, Svalof Weibull, Svalov, Sweden.

[3] Mr. C. Morgan, Crop Genetics Department, John Innes Centre, Norwich, UK.

[4] Mr. A. Keeling, Elsoms Seed, Spalding, UK.

[5] Mr. P. Dawson, A.L. Tozers Ltd, Cobam, Surrey, UK.

A.2.3 Results and Discussion

Regeneration medium containing 2 mg/l BAP was successfully used to regenerate shoots from cotyledonary explants of *B. oleracea* and *B. juncea*, however, *B. rapa* proved to be highly recalcitrant to *in vitro* regeneration on this medium. The literature supports this finding, and many groups have found *B. rapa* to be difficult to regenerate (Narasimhulu *et al,* 1988). A range of BAP and NAA concentrations were set up in

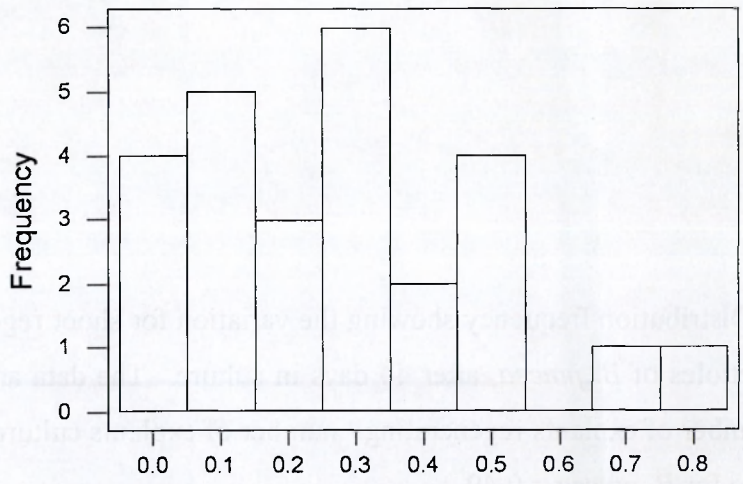
order to find an optimum combination for *B. rapa* regeneration. Results of this suggested 3 mg/l BAP plus 1.5 mg/l NAA to be the optimum conditions for regeneration of *B. rapa* (data not shown). Although shoot regeneration was achieved on this medium, a mass proliferation of callus also accompanied it; this was much greater than anything seen in the other brassicas. The frequency of shoot regeneration for the three *Brassica* species, using 2 mg/l BAP as the hormonal supplement for *B. oleracea* and *B. juncea* and 3mg/l BAP plus 1.5 mg/l NAA for *B. rapa*, are shown in Figure A.1 (after 23 days in culture) and Figure A.2 (after 44 days in culture). The majority of *B. rapa* genotypes were lost due to contamination following subculture to fresh medium after 23 day in culture, consequently the regeneration rates at 23 days are used to compared the three *Brassica* species.

Variation in shoot regeneration was noted within each of the Brassica species, with low, intermediate and high regenerating lines being observed. The variation was fairly continuous, and suggested that shoot regeneration may be controlled by a number of genes. The mean regeneration potentials from each species also showed there was variation between species. The overall mean shoot regeneration for *B. oleracea* (CC) was higher than *B. rapa* (AA) (58 % and 31 % respectively). The hybrid *B. juncea* (AABB) regenerated at a level higher than *B. rapa* (AA) (45 % and 31 % respectively). This supports the findings in the literature (discussed in Chapter 1 section 1.4.1.2), of *B. oleracea* being more responsive *in vitro* for shoot regeneration than *B. rapa*.

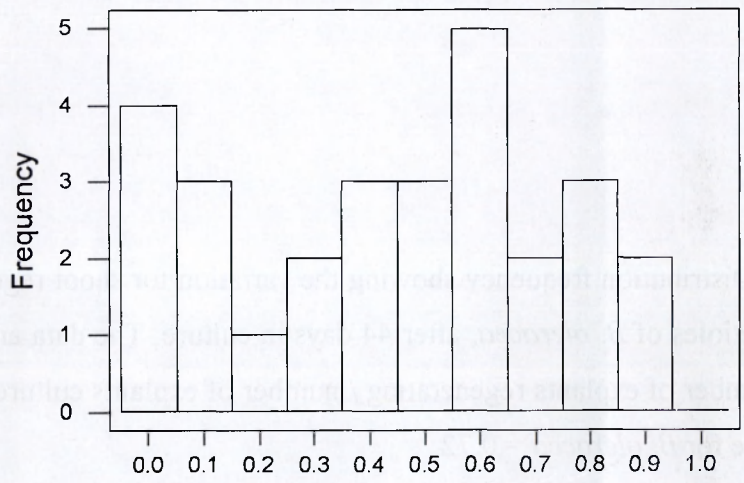
Figure A.1.1. Distribution frequency showing the variation for shoot regeneration from cotyledonary petioles of *B. rapa*, after 23 days in culture. The data are presented as frequencies (number of explants regenerating/ number of explants cultured). The mean regeneration rate for *B. rapa* = 0.31.

Figure A.1.2. Distribution frequency showing the variation for shoot regeneration from cotyledonary petioles of *B. juncea*, after 23 days in culture. The data are presented as frequencies (number of explants regenerating/ number of explants cultured). The mean regeneration rate for *B. juncea* = 0.45.

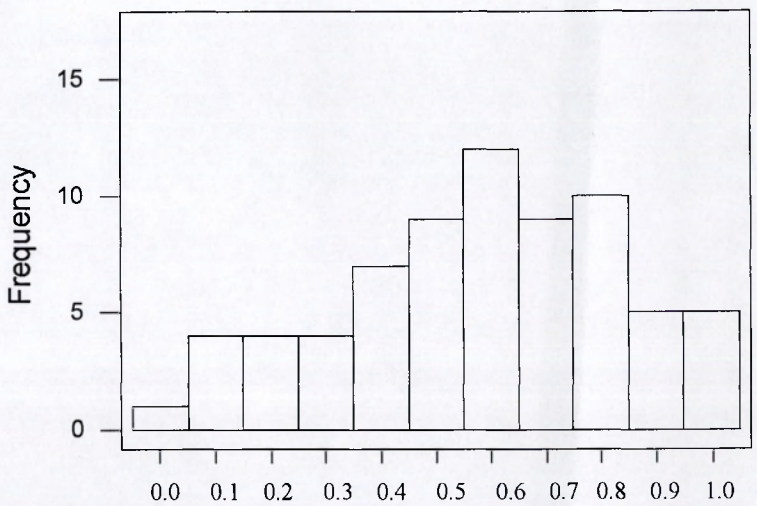
Figure A.1.3. Distribution frequency showing the variation for shoot regeneration from cotyledonary petioles of *B. oleracea*, after 23 days in culture. The data are presented as frequencies (number of explants regenerating/ number of explants cultured). The mean regeneration rate for *B. oleracea* = 0.58.



B. rapa



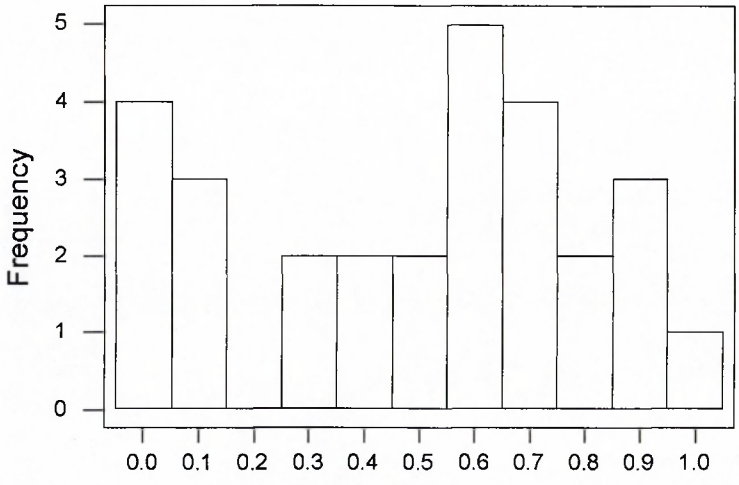
B. juncea



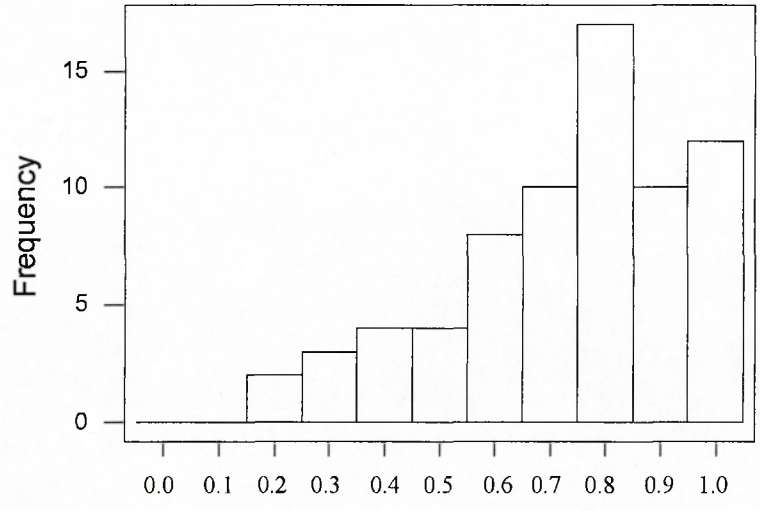
B. oleracea

Figure A.2.1. Distribution frequency showing the variation for shoot regeneration from cotyledonary petioles of *B. juncea*, after 44 days in culture. The data are presented as frequencies (number of explants regenerating / number of explants cultured). The mean regeneration rate for *B. juncea* = 0.49.

Figure A.2.2. Distribution frequency showing the variation for shoot regeneration from cotyledonary petioles of *B. oleracea*, after 44 days in culture. The data are presented as frequencies (number of explants regenerating / number of explants cultured). The mean regeneration rate for *B. oleracea* = 0.72.



B. juncea



B. oleracea

A.3 Trial Transformations

A.3.1 Introduction

Following the screen for shoot regeneration potential in the three *Brassica* species, high regenerating genotypes of *B. oleracea, B. juncea* and *B. rapa* were selected for trial transformations (high regenerating lines were those that regenerated shoots from > 70 % of explants).

A.3.2 Method

High regenerating genotypes were transformed with *A. tumefaciens,* strain LBA 4404 harbouring the plasmid p25Gi, which contains the genes *nptII* and *gus*. Full details of the transformation procedure are given in section 2.5. Co-cultivation and selection media used for *B. rapa* transformations were modified to contain 3 mg/l BAP and 1.5 mg/l NAA (instead of the 2 mg/l BAP used for *B. juncea* and *B. oleracea* transformations).

A.3.3 Results

B. rapa transformations

Two *B. rapa* genotypes were selected for transformation. Under transformation conditions the shoot regeneration potential of these genotypes was greatly reduced. The shoots that did regenerate were later shown to be non-transgenic

This suggests that either

1 The cells that were transformed (GUS positive callus was obtained for both genotypes) were not capable of regeneration *i.e.* cells targeted for regeneration may not be the same as those targeted for transformation events, and / or

2 The kanamycin selection levels were not high enough, and therefore regeneration favoured the non-transformed and less stressed cells (resulting in the production of escapes, *i.e.* green non-transformed shoots).

B. juncea transformations

Three *B. juncea* genotypes were selected for transformation; and each genotype produced a mass of green shoots. Histochemical analysis (to test for the presence of GUS expression – outlined in section 2.6) showed that the majority of these putative transgenics were indeed escapes, data not presented. The background level of resistance to kanamycin was much higher for *B. juncea* than that of *B. napus* (the brassica from which the modified transformation method was taken). Increasing selection to 150 mg/l (from 25mg/l) kanamycin was sufficient to allow for the selection of transformed non-chimeric transgenics. Transgenic plants were recovered from all three genotypes, with 0.06 to 1.3 % of explants producing transformed shoots.

B. oleracea transformations

Twenty high regenerating genotypes (that regenerated at a rate of $\geq$ 70 %) were selected for transformation. Transgenic plants were successfully obtained for 18 of the 20 genotypes. Transformation rates were determined by the percentage of inoculated explants that produced independent transgenic plants. Transformation rates of between 0.1 % to 7.7 %, were observed (see Figure A.3). The variation in transformation efficiencies observed for *B. oleracea* suggested that a high regeneration potential was not the only prerequisite for an efficient transformation system. Not only were variations in transformation efficiencies observed within *B. oleracea*, but variation was also observed between genotypes of the same subspecies. The cause of these variations could be a result of:

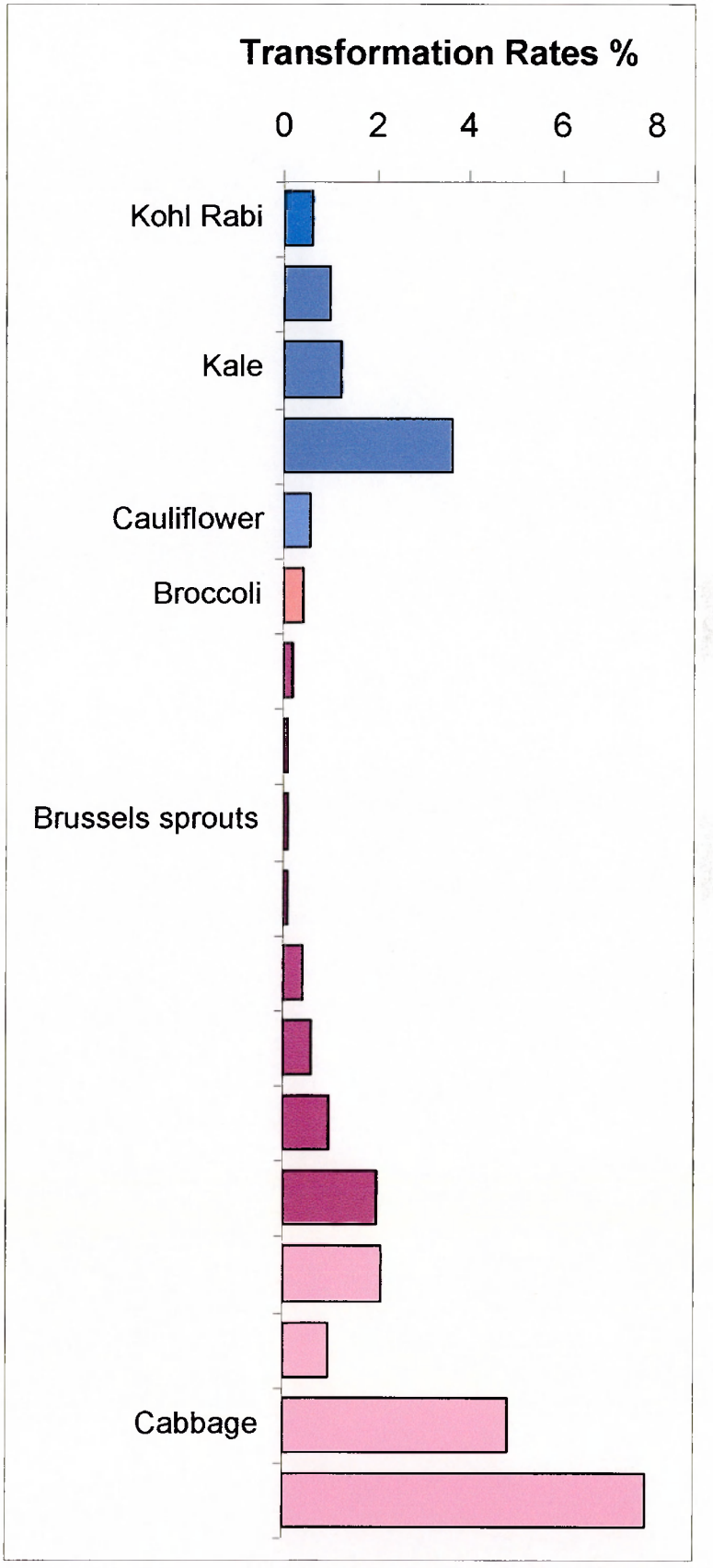
a) A genotype's susceptibility to *Agrobacterium*, be that due to hypersensitive responses or the inability to integrate T-DNA.

b) Transformation events targeting non-regenerable cells.

c) Inappropriate level of selection, supporting the regeneration of non-transformed cells (escapes).

Figure A.3 Variation in transformation efficiencies for six subspecies of *B. olercaea.*

The data are expressed as the percentage of explants, inoculated, that produced independent transgenic plants.


Transformation Rates %
0
2
4
6
8
Kohl Rabi
Kale
Cauliflower
Broccoli
Brussels sprouts
Cabbage

A.4 Background resistance to amino glycoside antibiotics

A common selectable marker gene used in many transformation systems is the *npt II* gene. This gene confers resistance to amino glycoside antibiotics, such as kanamycin. These amino glycoside antibiotics are able to bind to the 30S ribosomal subunits found in the mitrochondria and chloroplast of higher plants. This renders the ribosomal unit inactive and prevents the translation of mRNA into protein. The most dramatic and visible effect of this is chlorosis, or bleaching of the leaves due to lack of chlorophyll synthesis.

Selection protocols are highly species specific, with the type and concentration of antibiotic used varying considerably. Background resistance to kanamycin, the most commonly used amino glycoside antibiotic, was investigated in the diploid Brassicas.

A.4.1 Plant material

The diploid Brassica species, *B. rapa* (AA), *B. nigra* (BB) and *B. oleracea* (CC) were used in this study. Two genotypes of *B. rapa* and 2 genotypes of *B. nigra* (supplied courtesy of Dr. K.Vamling Svalof-Weibull, Sweden) along with 4 genotypes of *B. oleracea,* a kale, rapid cycling *albaglabra,* cabbage and broccoli genotype.

A.4.2 Methods

Seeds were surface sterilised as before and germinated in sigma pots containing germination medium (see also section 2.2). Five-day-old seedlings (with the roots removed) from the 8 Brassica genotypes were taken and transferred to sigma pots containing MS basal medium supplemented with either 0, 25, 50, 75, 100, 150 or 300

mg/l kanamycin. Explants were scored over 4 weeks for the number and colouration of newly emerging true leaves.

A.4.3 Results

The protocol was devised so that chlorosis, and therefore sensitivity to kanamycin could be screened without concern over variation in shoot regeneration (which would be the case if cotyledonary explants had been used to test for antibiotic sensitivity). Looking at meristematic growth also enabled a faster screening of the genotypes.

B. oleracea was the most sensitive of the three diploid species to kanamycin, with bleaching of emerging true leaves occurring at 25 mg/l whereas 200 to 300 mg/l and 300 mg/l were required to bleach out emerging true leaves of *B. rapa* and *B. nigra*, respectively. The *B. nigra* screened were highly heterogeneous, open-pollinated genotypes and within these genotypes segregation for kanamycin resistance was noted. Variation in background resistance to kanamycin, both between the diploid species, and the observation of segregation for resistance within *B. nigra,* would indicate that resistance to kanamycin might be under genetic control.

A.5 Conclusions from preliminary work

1 Shoot regeneration from cotyledonary petioles varied both between and within brassica species.

2 A high regenerating genotype is an advantage at the start of a transformation programme, but it is not the only limiting factor in a transformation system.

3 Variations in transformation efficiencies were observed within the species (perhaps due to different susceptibility to *Agrobacterium*, sensitivity to

kanamycin, or simply different cells are targeted for regeneration than those transformed).

4 Background resistance to kanamycin varied among the *Brassica* species with *B. nigra* ≥ *B. rapa* > *B. oleracea.*

Although not tested for background resistance, the results from the *B. juncea* transformation (where selection levels 6 times higher than *B. oleracea* were used) support the theory that resistance to antibiotics is under genetic control, and the absence of a C genome increases resistance to antibiotics.

This preliminary work sets the scene for the research presented within this thesis. This research uses a reference mapping population of *B. oleracea,* to investigate the genetic control of *in vitro* regeneration and susceptibility to *Agrobacterium tumefaciens.* It was not possible to investigate the genetic control of resistance to kanamycin within this population, as both parents (from which the doubled haploid population was derived) were highly sensitive to kanamycin and little variation was therefore observed across the population. This finding did, however, provide useful information for the adaptation of the transformation system outlined in section 2.5, and selection levels were reduced from 25 mg/l to 5 mg/l kanamycin, for initial transformations with genotypes from the DH mapping population.

Appendix B Preliminary regeneration trials

This section should be read in conjunction with section 3.2.2.2 (Regeneration within the DH mapping population). Distribution frequencies for both shoot and root regeneration from 4 trials (carried out at different time periods and with seed collected from different harvests) are presented. The trials varied in population size, and one trial was subject to unforeseeable environmental changes within the lifetime of the trial (when the culture room thermostat failed over a weekend period and temperatures of up to 30^0C were recorded). Explants were scored after 44 days in culture for the presence or absence of shoots and roots.

B.1 Shoot regeneration from cotyledonary petioles

Figures B.1 a-d, show the distribution frequencies across the DH population for shoot regeneration from cotyledonary petioles, when screened over 4 separate years. In order to show the conservation of ranking a selection of lines are highlighted to show shoot regeneration when screened over 4 separate years.

B.2 Shoot regeneration from hypocotyl explants

Figures B.2 a-d, show the distribution frequencies across the DH population for shoot regeneration from hypocotyl explants, when screened over 3 separate years (hypocotyl segments were not screened for shoot regeneration in the first year).

B.3 Root regeneration from cotyledonary petioles

Figures B.3 a-c, show the distribution frequencies across the DH population for root regeneration from cotyledonary petioles, when screened over 3 separate years.

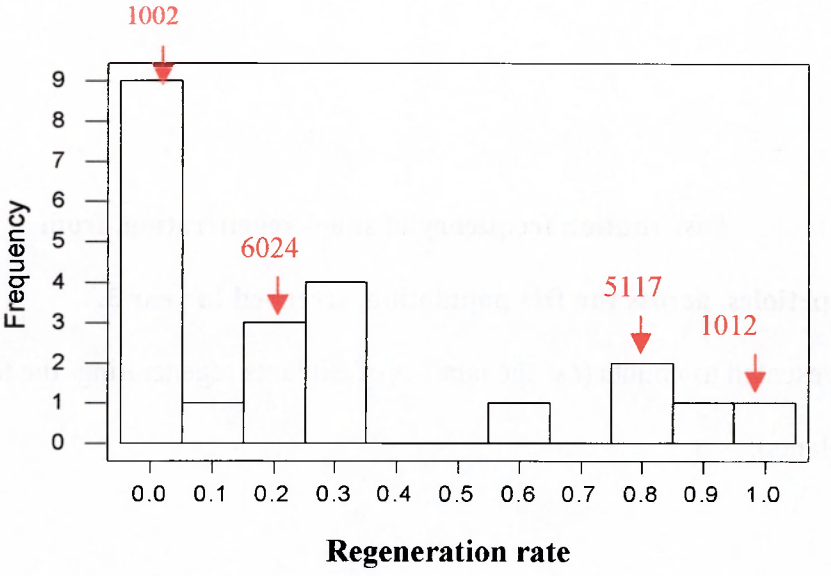
Figure B.1 a **Distribution frequency of shoot regeneration, from cotyledonary petioles, across the DH population, screened in year 1.**

The data are presented as counts (*i.e.* the number of explants regenerating/ the total number of explants).

Figure B.1 b **Distribution frequency of shoot regeneration, from cotyledonary petioles, across the DH population, screened in year 2.**

The data are presented as counts (*i.e.* the number of explants regenerating/ the total number of explants).

B.1 a


1002
6024
5117
1012
Frequency
0 1 2 3 4 5 6 7 8 9
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Regeneration rate

B.1 b

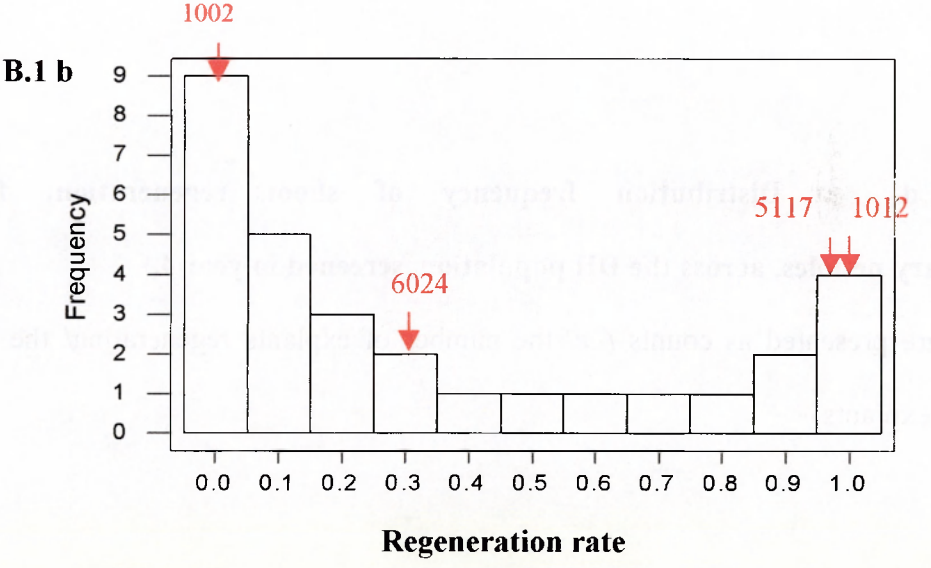
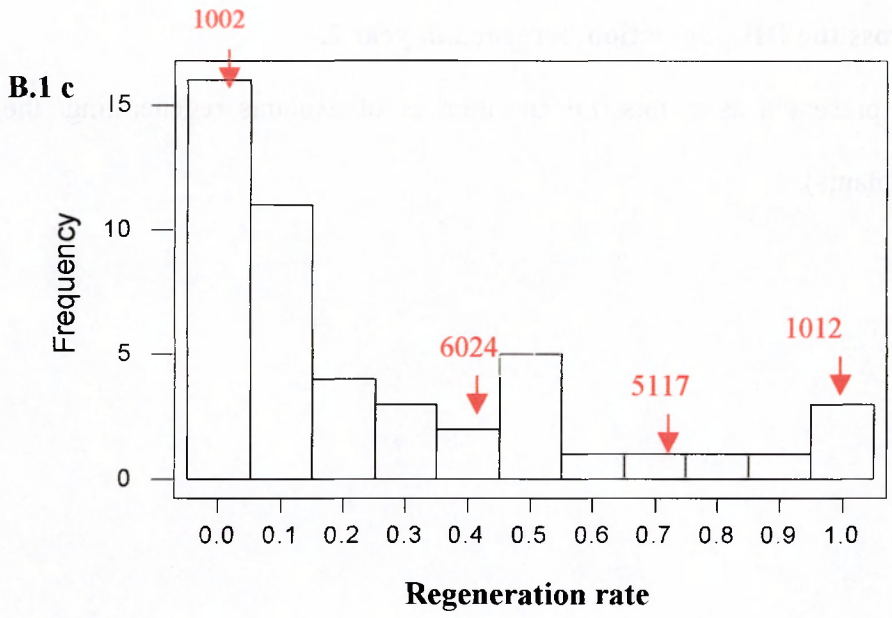

1002
5117
1012
6024
Frequency
0 1 2 3 4 5 6 7 8 9
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Regeneration rate

Figure B.1 c Distribution frequency of shoot regeneration, from cotyledonary petioles, across the DH population, screened in year 3.

The data are presented as counts (*i.e.* the number of explants regenerating/ the total number of explants).

Figure B.1 d Distribution frequency of shoot regeneration, from cotyledonary petioles, across the DH population, screened in year 4.

The data are presented as counts *(i.e.* the number of explants regenerating/ the total number of explants).


B.1 c
1002
6024
5117
1012
Frequency
0
5
10
15
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Regeneration rate

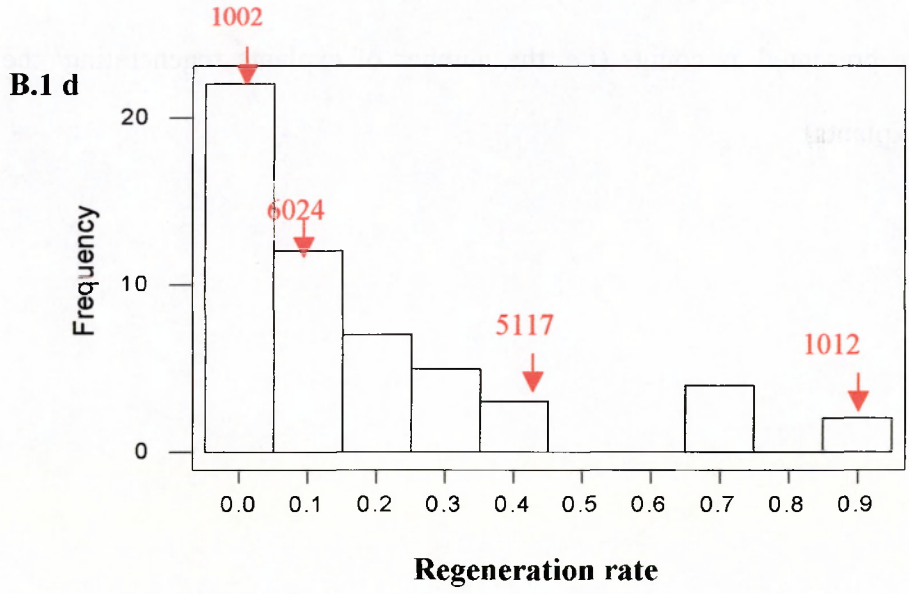

B.1 d
1002
6024
5117
1012
Frequency
0
10
20
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Regeneration rate

Figure B.2 a **Distribution frequency of shoot regeneration, from hypocotyl explants, across the DH population, screened in year 2.**

The data are presented as counts (*i.e.* the number of explants regenerating/ the total number of explants).

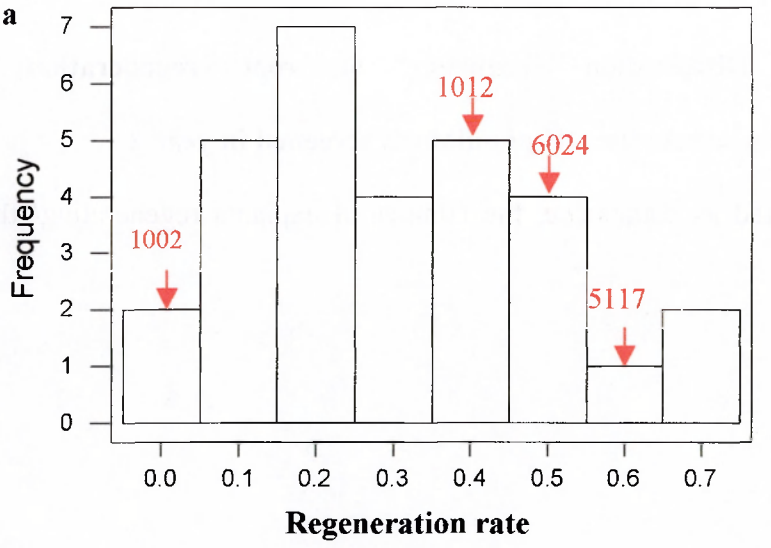
Figure B.2 b **Distribution frequency of shoot regeneration, from hypocotyl explants, across the DH population, screened in year 3.**

The data are presented as counts (*i.e.* the number of explants regenerating/ the total number of explants).

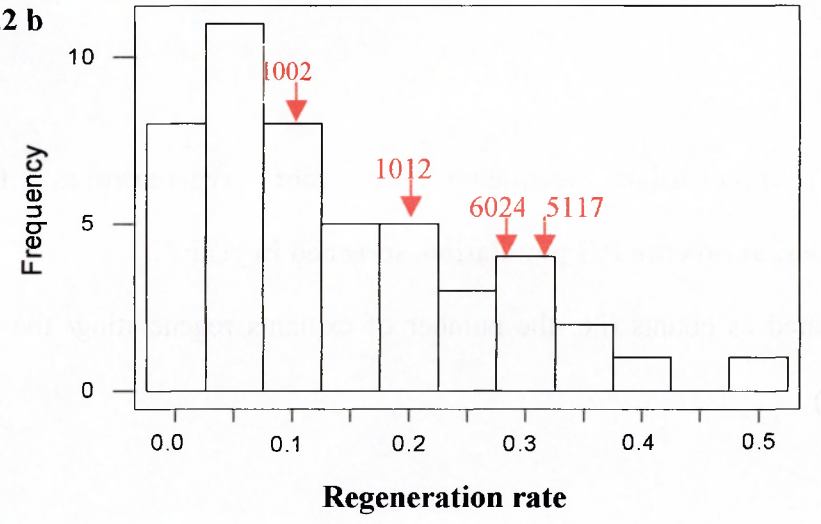
Figure B.2 c **Distribution frequency of shoot regeneration, from hypocotyl explants, across the DH population, screened in year 4.**

The data are presented as counts (i.e. the number of explants regenerating/ the total number of explants).

B.2 a


Frequency
0
1
2
3
4
5
6
7
1002
1012
6024
5117
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Regeneration rate

B.2 b


Frequency
0
5
10
1002
1012
6024
5117
0.0
0.1
0.2
0.3
0.4
0.5
Regeneration rate

B.2 c

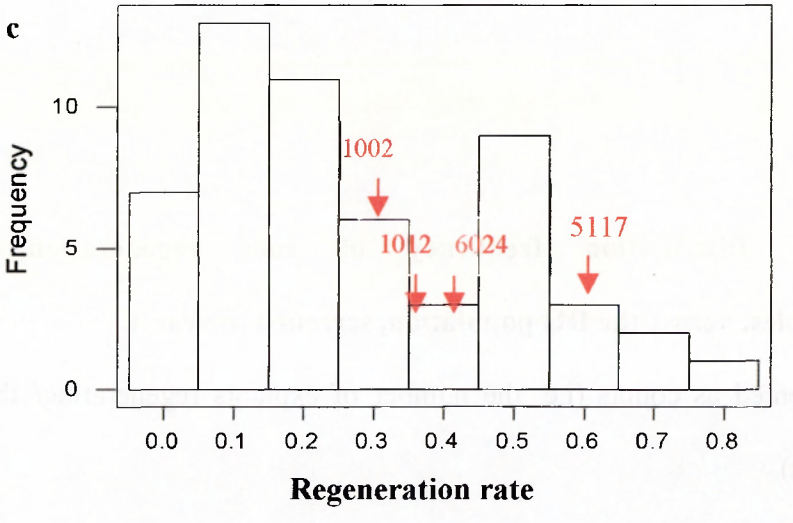

Frequency
0
5
10
1002
1012
6024
5117
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Regeneration rate

Figure B.3 a Distribution frequency of root regeneration, from cotyledonary petioles, across the DH population, screened in year 2.

The data are presented as counts (*i.e.* the number of explants regenerating/ the total number of explants).

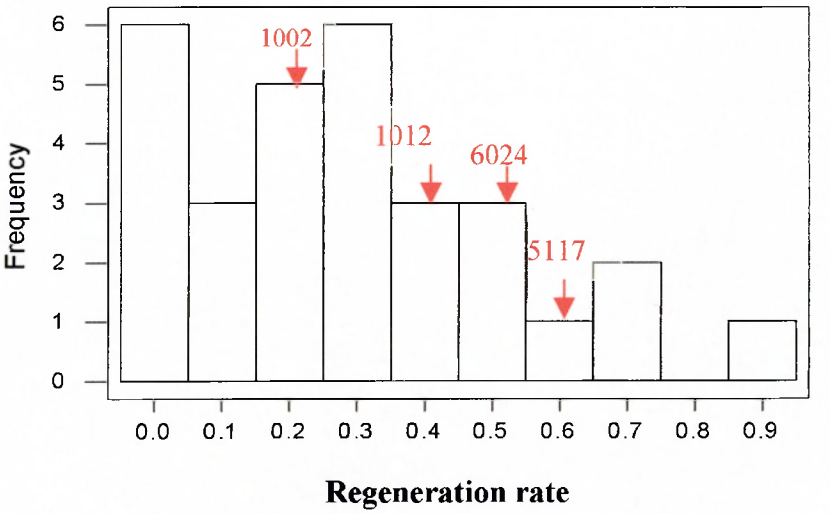
Figure B.3 b Distribution frequency of root regeneration, from cotyledonary petioles, across the DH population, screened in year 3.

The data are presented as counts (i.e. the number of explants regenerating/ the total number of explants).

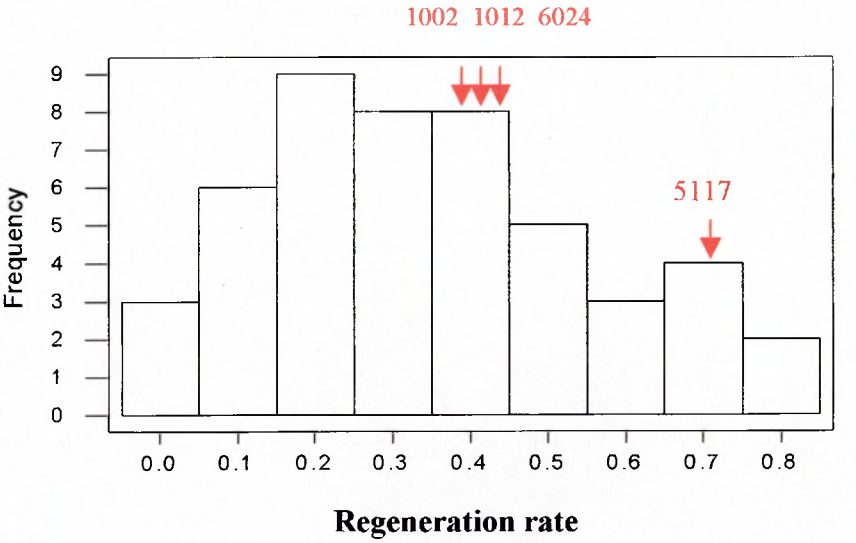
Figure B.3 c Distribution frequency of root regeneration, from cotyledonary petioles, across the DH population, screened in year 4.

The data are presented as counts (i.e. the number of explants regenerating/ the total number of explants).

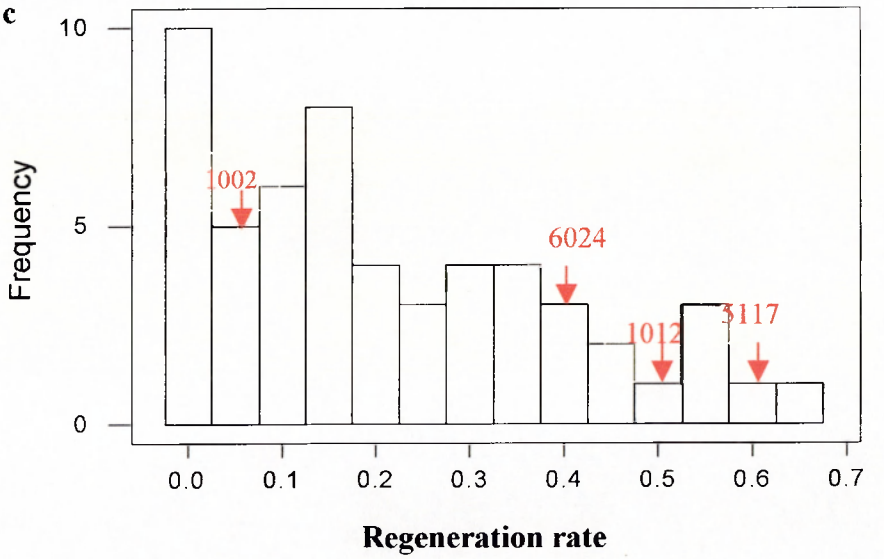
B.3 a


1002
1012
6024
5117
Frequency
0 1 2 3 4 5 6
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Regeneration rate

B.3 b


1002 1012 6024
5117
Frequency
0 1 2 3 4 5 6 7 8 9
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
Regeneration rate

B.3 c


1002
6024
1012
5117
Frequency
0 5 10
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7
Regeneration rate

The data clearly show that screening this population at different times, with increasing population sizes and in some cases varying environmental conditions, did not alter the profile of the distribution frequencies, and ranking of the regeneration responses remained the same. A significantly high correlation was observed between the data collected from each of the four screens. In other words, although overall regeneration rates went up and down as a population, the ranking within the population remained the same with the higher regenerating lines always remaining high, followed by the intermediate and then low regenerating lines.

APPENDIX C CALCULATIONS

C.1 Genetic component analysis

Genetic component analysis was carried out using values generated from the diallel analysis, using Genstat version 5.0, using formula described by Mather and Jinks (1987).

C.1.1 Formula

$E = E$

$D = Vp - E$

$H_1 = 4\bar{V}r + Vp - 4\bar{\bar{W}}r - (3n - 2/n)\,E$

$H_2 = 4\bar{V}r - 4V\bar{r} - (2(n^2 - 1)/n^2)\,E$

$F = 2Vp - 4\bar{\bar{W}}r - (2(n-2)/n)\,E$

Where*

E = The block interaction error (Mean Square value)

Vp = Variance of the parents

$\bar{V}r$ = Mean of Vr

$\bar{\bar{W}}r$ = Mean of Wr

$V\bar{r}$ = Variance of array means

* And these values were calculated as part of the diallel analysis using Genstat version 5.0.

$$\text{Broad-sense Heritability} = \frac{(\frac{1}{2} D + \frac{1}{2} H_1 - \frac{1}{4} H_2 - \frac{1}{2} F)}{(\frac{1}{2} D + \frac{1}{2} H_1 - \frac{1}{4} H_2 - \frac{1}{2} F + E)}$$

$$\text{Narrow-sense Heritability} = \frac{(\frac{1}{2} D + \frac{1}{2} H_1 - \frac{1}{2} H_2 - \frac{1}{2} F)}{(\frac{1}{2} D + \frac{1}{2} H_1 - \frac{1}{4} H_2 - \frac{1}{2} F + E)}$$

Genstat version 5.0® was used to calculate the Mean Square (MS) values, and the variance component was calculated using the formula described under Table C. The percentage of the total variation that could be attributed to the genotype, when used as the female (A) or male (B) donor, and the interaction between the two (AB) is given. More significantly, the percentage of the variation that is attributed to non-genetic effects, such as experimental errors and environmental effects is also calculated. Variation associated to such errors should ideally be less than 30 % if the experimental design is solid and the trait under investigation is under strong genetic control.

Table C Components of Variation

	Shooting from cotyledonary petioles		**Multiple shooting from cotyledonary petioles**		**Rooting from cotyledonary petioles**		**Shooting from hypocotyl segments.**	
Variance Component	**Value**	**%**	**Value**	**%**	**Value**	**%**	**Value**	**%**
σ^2 A	0.039	31.5	1.842	35.2	0.017	21.2	0.007	7.9
σ^2 B	0.042	33.9	1.845	35.3	0.025	31.3	0.012	13.5
σ^2 AB	0.025	20.2	0.382	7.3	0.014	17.5	0.011	12.4
σ^2	0.018	**14.5**	1.164	22.2	0.024	**30.0**	0.059	**66.3**
Total	0.124	100	5.233	100	0.080	100	0.089	100

Where: σ^2 AB = (AB MS - Error MS)/n

σ^2 A = (A MS – AB MS)/n b

σ^2 B = (B MS – AB MS)/n a

σ^2 = Error MS

A = Main effects attributed to Females (when lines are used as the female donor)

B = Main effects attributed to Males (when lines are used as the male donor)

AB = The interaction between A and B

MS = Mean Square value (calculated in Genstat version 5.0 ®)

n = number of replicates (in this case = 5), a = number of females (12) and b = number of males (12).